INHIBITION OF BACTERIAL FOODBORNE PATHOGENS ON THE SURFACES OF FRESH PRODUCE USING PLANT-DERIVED ANTIMICROBIAL ESSENTIAL OILS IN SURFACTANT MICELLES

A Dissertation

by

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ABSTRACT

This research was undertaken to: i) quantify numbers of native microbiota on leafy greens, jalapeno peppers, tomatoes, and cantaloupes; ii) study internalization in fresh produce with and without aid of temperature and pressure differential; iii) formulate essential oil component (EOC)-containing nano-micelles and analyze rheological and loading characteristics of particles; iv) identify the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of antimicrobial essential oil-containing micelles against *Escherichia coli* O157:H7 and *Salmonella enterica* serotype Saintpaul; and v) determine inactivation efficacy of EOC-containing micelles and other antimicrobial agents against *E. coli* O157:H7, *S.* Saintpaul, and epiphytic microbiota on surfaces of fresh produce.

Numbers of native microbiota on leafy greens obtained from South Texas in spring harvest seasons ranged from 0.7±0.0 to 6.2±0.1 $\log_{10}$ CFU/g. Higher counts of certain microbial groupings were observed with leafy green samples collected at higher ambient temperature. Native microbiota on surfaces of jalapeno pepper, tomato, and cantaloupe obtained from spring and fall harvest seasons were in the range of 0.2±0.0 to 3.9±0.7 $\log_{10}$ CFU/cm^2, 0.2±0.0 to 3.8±0.9 $\log_{10}$ CFU/cm^2, and 1.1±1.3 to 6.0±0.8 $\log_{10}$ CFU/cm^2, respectively. In general, stem scars of tomato and cantaloupe bore greater counts of native microbiota versus skins/rinds.

Dye penetration in intact and non-intact tomatoes with aid of temperature and pressure differential was 1.71±1.36 cm and 0.10±0.06 cm, respectively. The study of microbial internalization without aid of temperature and pressure differential showed

that internalization of *E. coli* K12 occurred through stem scar channels; however, *E. coli* K12 was unable to travel deeply in the stem.

The study of maximum additive concentration (MAC) of EOCs in surfactant micelles showed that sodium dodecyl sulfate (SDS) possessed the highest encapsulation efficiency among all tested surfactants. Carvacrol and eugenol encapsulated in SDS and CytoGuard LA20 (CG) micelles were most effective for pathogen inhibition in microbroth assay. In produce commodities, overall, encapsulated eugenol, free eugenol, chlorine, and empty micelles were similarly effective in reducing pathogens and native microbiota on tomato surfaces at 5 °C during 10 days of storage. At 15 °C, empty micelles were less effective than other antimicrobial treatments in reducing pathogens on tomato surfaces. Compared to encapsulated eugenol, free eugenol and empty micelles, decreased antifungal effect of chlorine was also observed at 15 °C in tomatoes. For spinach, encapsulated eugenol, free eugenol, and chlorine seemed to be similarly effective in reducing pathogen levels and were more effective than empty micelles and water at 5 and 15 °C. Overall, encapsulated eugenol and free eugenol were more effective than other treatments in reducing levels of aerobic bacteria and Enterobacteriaceae during storage at 5 and 15 °C. Excepting water, antifungal effects of all treatments did not differ during the entire storage period. The study suggests EOC-loaded micelles could be used as an alternative to conventional intervention methods for decontamination of fresh produce as well as increasing shelf life of fresh produce.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION

In recent years, foodborne illness outbreaks associated with fresh produce have increased markedly *(24, 87)*. Contamination of fruits and vegetables with pathogens can occur while growing in the fields, during harvesting, postharvest handling, processing and distribution. The source of contamination can be animals, soil, water, sewage, insects, and humans, etc. *(24)*. Pathogens associated with outbreaks in fresh produce include bacteria, viruses, and parasites; the pathogens of greatest concern include *Clostridium botulinum*, *Escherichia coli* O157:H7, *Salmonella enterica*, *Shigella* spp., *Listeria monocytogenes*, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, Hepatitis A virus, and Norwalk virus *(24)*. While a variety of bacterial pathogens have been linked to foodborne disease in connection with consumption of cross-contaminated produce, *E. coli* O157:H7 and serovars of *S. enterica* are of particular concern, given the estimated incidence of foodborne disease attributed to these agents and the severity of disease in consumers *(44, 45, 47, 50, 52, 54, 56)*.

A variety of pathogen intervention methods have been investigated to reduce pathogen loads in fresh and minimally processed produce *(82, 185, 239)*. Natural antimicrobials such as essential oil components (EOCs) have been reported to show effective activities against a wide range of pathogens *(13, 19, 217, 227)*. Unlike certain widely used antimicrobial agents (e.g. chlorine) that interact with organic matter producing toxic substances (e.g. chloramine, trihalomethanes) *(265)*, EOCs do not produce undesirable by-product from interaction with organic substances *(15)*. Also,

EOCs are generally recognized as safe (GRAS) by the United States Food and Drug Administration based on 21 Code of Federal Regulation (CFR) 182.20 *(95).* However, EOCs are normally hydrophobic and possess low solubility in aqueous phases, resulting in a requirement of high concentration to exert inhibitory effects against foodborne pathogens *(247).*

Encapsulation of EOCs in surfactant micelles has been reported to improve dispersion of EOCs in aqueous phases resulting in enhanced delivery of EOCs to microbial pathogens *(107, 189).* However, very few studies on the antimicrobial efficacy of nanoencapsulated EOCs in surfactant micelles have been done in food systems, especially fresh produce. Thus, the objectives of this study were to i) quantify numbers of native microbiota on leafy greens, jalapeño peppers, tomatoes, and cantaloupes, ii) study internalization in fresh produce with and without aid of temperature and pressure differential, iii) formulate EOC-containing nano-micelles and analyze rheological and loading characteristics of particles, iv) identify the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of antimicrobial essential oil-containing micelles against *E. coli* O157:H7 and *S.* Saintpaul, and v) determine inhibition efficacy of essential oil-containing micelles against *E. coli* O157:H7, *S.* Saintpaul, and native microbiota on surfaces of fresh produce.

CHAPTER II

NATIVE MICROBIOTA OF FRESH PRODUCE

Fresh produce surfaces harbor a large variety of microbes including bacteria, yeasts, molds and viruses. Bacteria found on surfaces of produce include both Gram-negative and -positive organisms *(101, 140)*. Additionally, diverse molds and yeasts (e.g. *Botrytis cinerea, Rhizopus* spp., *Alternaria* spp., *Penicillium* spp., *Cladosporium* spp., *Fusarium* spp., *Trichoderma* spp., *Aureobasidium* spp., *Catellospora* spp., etc.) are reported to inhabit produce surfaces *(69, 230)*. The majority of Gram-negative bacteria present on the surface of fresh fruits and vegetables include members of the families Pseudomonadaceae (e.g. *Pseudomonas*), Enterobacteriaceae (e.g. *Erwinia*, *Enterobacter*, *Citrobacter*), and Achromobacteriaceae *(140, 153, 204)*. Predominant Gram-positive bacteria on the surface of fresh produce include Lactic Acid Bacteria (LAB) and members of families Bacillaceae, Micrococcaceae, Corynebacteriaceae (e.g. *Corynebacterium*) and Staphylococcaceae *(140)*. In the same produce commodity, the types and proportions of microorganisms on different tissue types can vary *(204)*. For example, Gram-negative bacteria are more abundant on the surfaces of outer leaves, while the higher number of LAB are found on the interior of heads than from the exterior *(153)*.

The internal tissues of healthy fruits and vegetables are normally assumed to be sterile; however, the presence of low number of microorganisms in the internal tissue has been reported *(153, 201, 202)*. Samish et al. *(203)* studied the presence of bacteria in the internal tissues of tomatoes and reported that 45 of 62 tomatoes contained members

of the family Pseudomonadaceae (few *Pseudomonas*). Bacteria in the family Enterobacteriaceae (five bearing 5 *Aerobacter cloacae* and one *Escherichia intermedia*) were found in 6/62 tomatoes while members of the families Micrococcaceae, Achromobacteriaceae (*Flavobacterium*) and Corynebacteriaceae were found in 5, 2, and 1 tomatoes, respectively *(203)*. They reported that tomatoes containing bacteria in internal tissues did not differ from bacteria-free tomatoes in appearance, flavor, or acidity *(203)*. Tomato stem scars contained low concentrations of *Bacillus*, *Micrococcus*, and yeasts and molds *(204)*. Bacterial population gradients were observed within internal tissues of produce . In cucumbers, bacteria are more abundant in the internal tissues close to the periphery than the central core *(204)*. In tomatoes, the concentration of bacteria is highest in the tissues around the stem scar, while bacterial numbers decrease towards the stylar end and near the fruit periphery *(204)*. Pods of green beans, broad beans and garden peas contained higher bacterial number versus seeds *(204)*.

Certain bacterial microorganisms such as *Pseudomonas*, *Erwinia*, *Enterobacter*, *Xanthomonas*, *Pectobacterium*, and *Lactobacillus* can cause spoilage of fresh produce. Yeasts (e.g. members of *Cryptococcus*, *Rhodotorula*, and *Saccharomyces*) and molds (e.g. *Penicillium* and *Aspergillus* spp., *Eutorium* spp., *Alternaria* spp., *Cladosporium* spp., and *Botrytis cinerea*) are also involved in spoilage of fresh fruits and vegetables *(140, 229, 230)*. Fungi can penetrate through intact plant surfaces and natural openings using appressorium and penetration pegs *(151)*. Unlike fungi, bacteria do not possess mechanisms to penetrate protected tissues (e.g. cuticle, epidermis, bark), penetration is therefore only limited to wounds or other unprotected tissues (e.g. stomata, lenticels,

stem scars, etc.) *(214)*. Spoilage bacteria can be transmitted to fresh produce surface via rain, irrigation water, insects, soil, animals, etc. With adequate moisture or the presence of fresh wounds, bacteria might survive or grow on the plant surface. Since internal tissue contains high moisture, bacteria can grow or survive in the plant tissue. Some spoilage bacteria (e.g. *Erwinia carotovora*) obtain nutrients through pectinolytic enzymes that hydrolyze pectic substance components of the middle lamella and primary cell wall of plant cells *(3, 123, 140)*. Examples of microorganisms that cause spoilage in fresh produce are listed in Table 2-1.

TABLE 2-1. Selected microorganisms implicated in spoilage of fresh produce.

Implicated Microorganism	Spoilage Type	Affected Produce
Bacteria		
Erwinia carotovora	Bacterial soft rot	Leafy crucifers, lettuce, endives, parsley, celery, carrots, onions, garlic, tomatoes, beets, peppers, cucumbers
Pseudomonas chicorii	Bacterial zonate spot	Cabbage and lettuce
Pseudomonas marginalis	Soft rot of vegetables	Lettuce and others
Pseudomonas tomato	Bacterial specks	Tomatoes
Pseudomonas syringae	Diseases in soybeans	Soybeans
Xanthomonas campestris	Black rot	Cabbage and cauliflower
Fungi		
Alternaria tenuis	Alternaria rot	Citrus fruits
Alternaria brassicola	Alternaria rot	Leafy crucifers

TABLE 2-1. Continued.

Implicated Microorganism	Spoilage Type	Affected Produce
Botrytis cinerea	Grey mold rot	Grapes, leafy crucifers, lettuce, onions, garlic, asparagus, pumpkin, squash, carrots, celery, sweet potatoes other vegetables
Aspergilus niger	Black rot	Onions, cabbage
Cladosporium cucumerinum	Scab	Cucumber and pumpkin
Cladosporium herbarum	*Cladosporium* rot	Cherries, peaches
Fusarium spp.	Dry rot	Potatoes
Geotrichum candidum	Sour rot	Tomatoes, citrus fruits
Penicillium digitatum	Blue mold rot	Citrus fruits

From *(133, 229)*

Certain native microbiota exert beneficial effects in inhibiting plant/human pathogen attachment, survival and multiplication on produce surfaces *(140)*. Ukuku et al. *(233)* studied inhibition of *L. monocytogenes* by native microbiota on whole and fresh cut cantaloupes treated and not treated with water, 70% ethanol, or 200 ppm chlorine *(233)*. After treatments, whole cantaloupes were inoculated with 7 $\log_{10}$ CFU/ml of *L. monocytogenes* and fresh cut cantaloupes were inoculated with 3.48 $\log_{10}$ CFU/g *(233)*. Samples were stored at 5, 10 and 20°C for 15 days. Overall, levels of *L. monocytogenes* declined over 15 days of storage at all tested temperatures; however, more rapid decline was observed with samples treated with water, 70% ethanol, or 200 ppm chlorine *(233)*. This suggests that native microbiota on whole and fresh cut cantaloupes could inhibit

attachment of *L. monocytogenes* on cantaloupe rinds and survival of *L. monocytogenes* on rinds and fresh cut samples *(233)*. Janisiewicz et al. *(131)* reported that *Pseudomonas syringiae* (2.4 x 10^8 CFU/ml) inoculated in wounds of apple prevented growth of *E. coli* O157:H7 *(131)*. However, two log increase was observed with wounds not treated *P. syringiae (131).* The possible mechanisms of antagonistic microorganisms may include: 1) outcompeting pathogens for space and nutrients; 2) production of antagonistic compounds (e.g. bacteriocins, hydrolytic enzymes, antibiotics) that affect growth of pathogens; 3) triggering of defense responses in the host, resulting in enhanced pathogen resistance *(90, 138).*

CHAPTER III

PRODUCE CONTAMINATION

3.1 Foodborne Illnesses Associated with Fresh Produce

The U.S. Centers for Disease Control and Prevention (CDC) estimated that 47.8 million illnesses, 127,839 hospitalizations and 3,037 deaths occur annually due to known and unspecified foodborne microbial pathogens *(57).* Additionally, microbial foodborne pathogens transmitted by fresh and minimally processed produce have been repeatedly identified to cause human foodborne disease in the U.S. *(48, 179)*. Over the past four decades, the number of foodborne illness outbreak associated with fresh produce and reported to CDC has increased *(21, 154, 215)*. For example, the foodborne illness outbreaks increased from 0.7% in the 1970s to 6% in the 1990s. The increase in outbreak numbers could be partly due to improved surveillance for human pathogens *(92)*, increased consumption of raw fruits and vegetables *(231)*, and improved pathogen detection and diagnostic methods *(135)*. From 2004 to 2012, norovirus contributed to the most outbreaks (223 outbreaks) related to fresh produce, followed by *Salmonella* enterica. (71 outbreaks), enterohemorrhagic *E. coli* (46 outbreaks), and *Campylobacter* spp. (9 outbreaks) *(38)*. Leafy greens have been the produce commodity type most frequently implicated in produce outbreaks *(39)*. From 1996 to 2006, leafy greens have contributed to 34% of all fresh produce outbreaks *(39)*. Examples of fresh produced-related foodborne illness outbreaks occurring in North America are listed in Table 3-1.

TABLE 3-1. Reported foodborne disease outbreaks associated with produce.

Implicated Pathogen	Year	Location	Cases	Produce Commodity Associated with Outbreak
Salmonella Poona	2015	Multistate, USA	888	Imported Cucumbers
Salmonella Newport	2014	Multistate, USA	275	Cucumbers
Salmonella Enteritidis	2014	Multistate, USA	115	Bean sprouts
Cyclospora cayetanensis	2014	Multistate, USA	304	Cilantro
Escherichia coli O121	2014	Multistate, USA	19	Clover sprouts
Salmonella Saintpaul	2013	Multistate, USA	84	Cucumbers
Escherichia coli O157:H7	2013	Multistate, USA	33	Ready-to-eat salads
Salmonella Braenderup	2012	Multistate, USA	127	Mangoes
Salmonella Typhimurium *and Salmonella* Newport	2012	Multistate, USA	261	Cantaloupes
Escherichia coli O26	2012	Multistate, USA	29	Clover Sprouts
Escherichia coli O157:H7	2011	Multistate, USA	58	Romaine lettuce
Escherichia coli O157:H7	2011	Multistate, USA	8	Hazelnuts
Salmonella Enteritidis	2011	Multistate, USA	43	Turkish pine nuts
Listeria monocytogenes	2011	Multistate, USA	147	Whole cantaloupes
Salmonella Agona	2011	Multistate, USA	106	Imported papaya
Salmonella Enteritidis	2011	Multistate, USA	25	Alfafa and spicy sprouts
Salmonella Panama	2011	Multistate, USA	20	Cantaloupes
Salmonella I	2010	Multistate, USA	140	Alfalfa sprouts
Salmonella Newport	2010	Multistate, USA	44	Alfalfa sprouts
Escherichia coli O145	2010	Multistate, USA	26	Romaine lettuce
Salmonella Montevideo	2010	Multistate, USA	272	Red and black peppers
Salmonella Saintpaul	2009	Multistate, USA	235	Alfafa sprouts
Salmonella Litchfield	2008	Multistate, USA	51	Cantaloupes

TABLE 3-1. Continued.

Implicated Pathogen	Year	Location	Cases	Produce Commodity Associated with Outbreak
Salmonella Poona	2000	Multistate, USA	N/A	Cantaloupes
Salmonella Enteritidis	2000	Alberta and Saskachewan, Canada	8	Alfafa sprouts
Salmonella Enteritidis	2000	California	45	Mung beans
Cyclospora cayetanensis	1999	Ontario, Canada	104	Blackberries
Salmonella Havana	1998	California and Arizona	18	Sprouts
Escherichia coli O157:H7	1998	Indianapolis	33	Coleslaw
Escherichia coli O157:H7	1998	Wisconsin	47	Fruit salad
Cryptosporidium parvum	1997	Washington	54	Green onions
Cyclospora cayetanensis	1997	Multistate, USA	>308	Basils
Campylobacter jejuni	1996	Oklahoma	14	Lettuce
Escherichia coli O157:H7	1995	Maine	30	Iceberg lettuce
Shigella flexneri 6A	1994	Multistate, USA	72	Green onions
Escherichia coli O157:H7	1993	Oregon	9	Cantaloupe
Escherichia coli O157:H7	1991	Massachusetts	23	Apple
Hepatitis A virus	1988	Scotland	5	Raspberries
Salmonella Typhi	1989	New York	46	Oranges
Clostridium botulinum	1987	New York	3	Chopped garlic in oil
Norwalk virus	1987	United Kingdom	206	Melons
Shigella sonnei	1987	Sweden	15	Watermelons

From *(58, 93)*

Produce-related foodborne diseases can cause enormous economic impact including medical expenses, lost wages, damage control costs for product recall and disposal of affected products, and lost of production time *(205)*. The outbreak history can even adversely affect the entire segment of the produce industry resulting in decreases in sales and consumption of the products due to lost confidence in product safety *(205)*. The *E. coli* O157:H7 2006 outbreak in spinach dramatically decreased the U.S. export markets to Canada, the largest importer of the U.S. leafy greens *(39)*. Canada temporarily stopped importing spinach from the U.S. and the sales were low even after the market resumed *(39)*.

3.2 Potential Routes of Pathogen Contamination in Fresh Produce

Human pathogens can come in contact with fresh produce commodities and then strongly attach and adhere *(232)* or even form biofilm on produce surfaces *(257)*. Once colonization of pathogens on fresh produce occurs, removing pathogens from contaminated produce become very difficult *(106)*. Factors affecting survival or growth of pathogens on produce surfaces include the type of pathogen, type of produce commodity, pre-harvest and post-harvest environmental conditions (e.g. temperature, humidity, irrigation method), nutrient availability *(92)*, and interaction of pathogens with microbes that colonize plant surfaces (epiphytes) *(62)*. Fresh produce can become contaminated at any point during growing, harvesting, and postharvest handlings *(24)*. Figure 3-1 further demonstrates possible mechanisms/routes that fresh produce commodities can become contaminated with human pathogens.

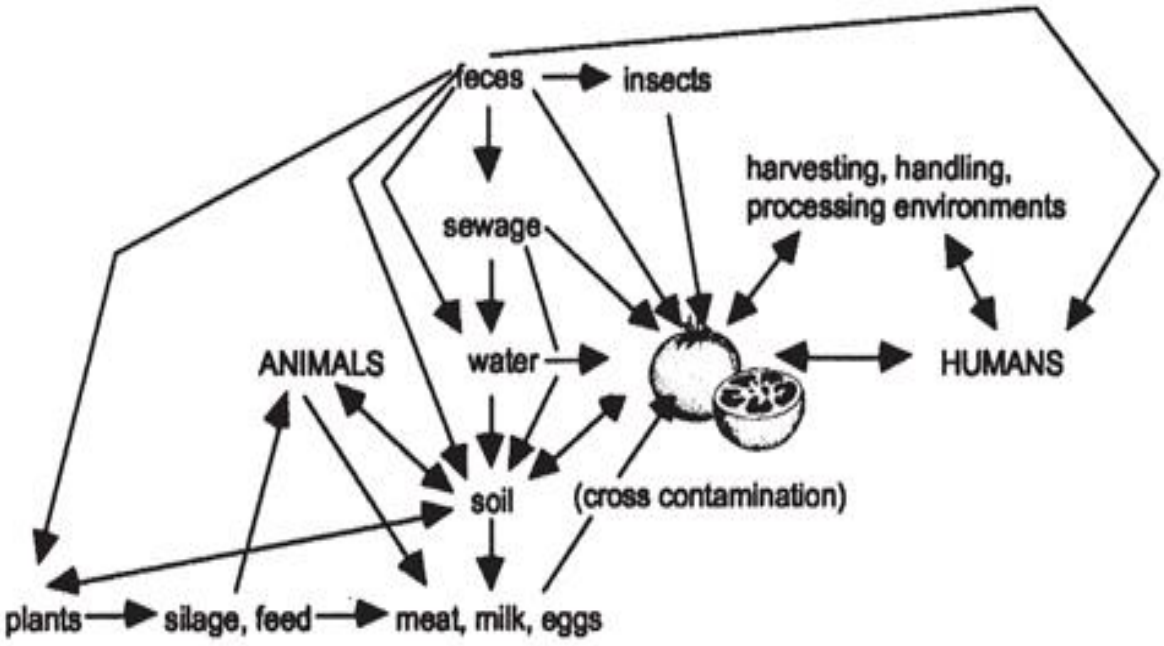

FIGURE 3-1. Possible mechanisms/routes that fresh produce commodities can become contaminated with human pathogens *(24)*.

3.2.1 Pre-harvest Sources of Pathogen Contamination

Pre-harvest sources of pathogen contamination in fresh produce include animal feces, soil, irrigation water, fertilizer, air (dust), animals, and human handling, rain *(24)*.

During growing in the field, soil can be a contamination agent for fresh produce *(119)*. Since soil can be contaminated with fecal matter, contaminated water, and improperly treated fertilizers etc., it can harbor a variety of microorganisms including human pathogens *(119)*. Thus, transfer of pathogens from soil to produce during growing can cause produce to become contaminated *(167)*. Generally, moist cold soil

with neutral or slightly alkaline pH favors microbial survival more efficiently than dry soil *(238)*.

Manure has been widely used to fertilize and maintain soil fertility and quality for growing crops due to economical and environmental benefits *(122)*. Nevertheless, manure can harbor human pathogens such as Shiga toxin-producing *E. coli*, *Salmonella*, *Campylobacter*, and *Listeria*, etc. *(173)*. Thus, the application of manure in agricultural lands can introduce pathogens to the food chain, and the risk is highest when crops are consumed raw (e.g. salads, fresh fruits and vegetables) *(173)*. Factors affecting prevalence and levels of human pathogens in livestock manure can include fecal shedding from livestock, age of livestock (pathogen levels higher in feces of young livestock), dietary changes, and animal stress *(173)*. Kim et al. *(143)* conducted a study to determine factors affecting growth of *E. coli* O157:H7 in dairy compost (treated soil amendment). Regrowth of *E. coli* O157:H7 occurred when the background microbiota level of manure was low (2.3 to 3.9 $\log_{10}$ CFU/g) and suppression of pathogen regrowth occurred when the background manure microbiota level was higher (~6.5 $\log_{10}$ CFU/g). Moisture content, pH, temperature also affect survival and growth of *E. coli* O157:H7 in cattle manure *(25)*. When the external composting temperature was 50 °C, 4.0 to 7.0 $\log_{10}$ CFU/g of *E. coli* O157:H7 were inactivated to undetectable within 7-14 days *(25)*. Thus, cattle manure contaminated with *E. coli* O157:H7 should be composted at the minimum temperature of 50 °C for 1 to 2 weeks to inactivate the pathogen *(25)*.

Irrigation water is an important source of pathogen contamination in fresh produce *(135)*. When irrigation water comes into contact with fresh produce, the water

quality dictates the potential for the pathogen contamination *(99)*. Irrigation source and method plays also an important role in transferring human pathogen to fresh produce *(240)*. Irrigation water can originate from many sources including municipal water, rain water, groundwater, surface water (open canal, ponds, reservoirs, and lakes), and waste water *(240)*. Generally, municipal water possesses the best microbial quality (potable quality), followed by groundwater, rainwater, and surface water *(240)*. Wastewater is of the lowest quality since it is mostly discharged without any treatment, thus posing health hazard risks *(254)*. Commonly used irrigation methods include subsurface (e.g. drip irrigation) and surface irrigation (e.g. flood irrigation, furrow irrigation, and sprinkler irrigation) *(9, 105)*. Complete coverage of the soil surface is obtained by flood irrigation resulting in direct farm worker exposure more than other methods. When contaminated water is used, flood irrigation therefore can lead to the greatest health hazards risk to growers and consumers *(240)*. Aerosols containing pathogens from sprinkler irrigation can spread through wind. With low wind velocity, Enterobacteriaceae could be detected at a distance of 60 to 160 m downwind from the sprinkler *(5)*. Unlike surface irrigation, subsurface irrigation allows water to be in contact with plant roots *(112)*. Thus this method is generally safer in terms of protecting pathogen transmission to farmers and consumers *(240)*. It has been reported that human pathogens can survive for a certain period of time in irrigation water *(129, 167, 176, 178)*. Olivera et al. *(176)* reported that *E. coli* O157:H7 could be transferred from soil irrigated with contaminated water or fertilized with contaminated soil to edible parts (inner and outer leaves) of lettuce.

3.2.2 Harvest and Post-harvest Sources of Pathogen Contamination

Harvest and post-harvest sources of pathogen contamination in fresh produce can include harvesting equipment, feces, human handling, animals, air (dust), wash and rinse water, packing and processing equipment, ice, transport vehicle, improper temperature, improper storage, improper packaging *(24)*.

During harvest, harvesting equipment can be implicated as a source of human pathogen contamination *(205)*. Harvesting equipment is designed to be used for specific location of the produce commodity. For example, baby spinach is commercially harvested using lawn mower-type machines that can introduce human pathogens to spinach due to contact with soil and manure *(35)*. Harvesting lettuce in the field is performed by cutting and coring lettuce head to reduce shipping waste and maximize production yield *(81, 165)*. However, this can also introduce pathogen contamination to lettuce *(81)*. The device is composed of a stainless steel wedged shaped blade which is used to cut the lettuce stem near the soil surface and a cylindrical coring ring which is inserted around the stem of the lettuce head to remove the core *(81)*. McEvoy et al. *(165)* conducted a study to examine potential growth of *E. coli* O157:H7 on field-cored lettuce using a coring knife inoculated with 2 x 10^5 cells of *E. coli* O157:H7 *(165)*. At 30 °C, growth increased significantly ($p<0.001$) by more than 2.0 log CFU/g from 0 to 8 hr *(165)*. However, significant growth was not observed ($p>0.05$) when cored lettuce samples were held at 5 °C. The study suggests that prompt chilling of freshly cored lettuce and prevention of knife contamination can help to ensure safety of the filed cored lettuce *(165)*. Yang et al. *(256)* also reported transfer of *E. coli* O157:H7 from artificially

contaminated soil to iceberg lettuce via coring knife as affected by degree of blade contact (stem, medium, and heavy), *E. coli* O157:H7 was detected on first and second sequentially cut lettuce heads using medium contact between knife blade and edible tissues *(256)*. *E. coli* O157:H7 was also detected in cut head lettuce using the heavy-contact cutting.

Worker hygiene also largely dictates microbiological safety of fresh produce *(86)*. Ill or infected workers can serve as a primary source of human pathogen such as norovirus, hepatitis A virus, *Shigella*, and *Salmonella*, etc. *(154)*. Since workers may come from diverse backgrounds, inferior personal hygiene practices may result in propagation of human pathogens *(35)*. Thus, proper training programming to follow good hygienic practices must be established and performed *(86)*. For example, employees should be trained to use proper hand-washing technique, become familiar with typical signs and symptoms of infectious diseases, and understand the importance of using toilet facilities. Not only does good hygiene protect workers from illnesses, but it also reduces potential fresh produce contamination which could cause widespread foodborne outbreaks if consumed. In 1995, a *Vibrio cholerae* outbreak in cantaloupes occurred in California *(1)*. The source of pathogen contamination was identified to be the worker who had sliced the cantaloupe had returned from a 3-week visit to Guatemala *(1)*. The worker did not develop any gastrointestinal illnesses during or in 7 days after return, thus suggesting secondary transmission of cholera linked to an asymptomatic food handler *(1)*.

3.3 Preventive Measures for Controlling Hazards in Produce

Approaches for preventing contamination in produce are warranted and include effective management and intervention strategies for growing, handling, distributing, and preparing fresh produce. The approaches include but are not limited to Good Agricultural Practices (GAPs), Current Good Manufacturing Practices (cGMPs), and Hazard Analysis and Critical Control Points (HACCP) programs *(135)*.

3.3.1 Good Agricultural Practices (GAPs)

In October 1997, the plan entitled "Initiative to Ensure the Safety of Imported and Domestic Fruits and Vegetables" was announced by President Clinton to provide further assurance that fruits and vegetables consumed by Americans meet the optimum standard *(91)*. In response to this initiative, since 1998, the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) have issued guidance on good agricultural practices (GAPs) for the produce industry; the guidance entitled "Guidance for Industry—Guide to Minimize Food Safety Hazards for Fresh Fruits and Vegetables" *(91)*. This guidance document is not a regulation, and thus does not have force and effect of law *(86)*. If applied properly to fruit and vegetable production, the guide will help minimize food safety hazards from microbial pathogens. GAPs are scientifically based on eight principles as follows *(86)*:

Principle 1: Preventing microbial contamination of fresh produce is favored over relying on corrective actions once contamination has occurred.

Principle 2: To minimize microbial food safety hazards in fresh produce, growers, packers, or shippers should use good agricultural and management practices in those areas over which they have control.

Principle 3: Fresh produce can become microbiologically contaminated at any point along farm-to-table food chain. The major source of microbial contamination with fresh produce is linked to human or animal feces.

Principle 4: Whenever water comes in contact with produce, its source and quality dictates potential for contamination. Minimize the potential of microbial contamination from water used with fresh fruits and vegetables.

Principle 5: Practices using animal manure or municipal biosolid wastes should be managed closely to minimize the potential for microbial contamination of fresh produce.

Principle 6: Worker hygiene and sanitation practices during production, harvesting, sorting, packing, and transport play an important role in minimizing the potential for microbial contamination of fresh produce.

Principle 7: Follow all applicable local, state, and Federal laws and regulations, or corresponding or similar laws, regulations, or standards for operators outside the U.S., for agricultural practices.

Principle 8: Accountability at all levels of the agricultural environment (farm, packing facility, distribution center, and transport operation) is important to a successful food safety program. There must be qualified personnel and effective monitoring to

ensure all elements of the program function correctly and to help track produce back through the distribution channels to the producer.

3.3.2 Current Good Manufacturing Practices (cGMPs)

The cGMPs are in 21 CFR 110 and provide guidelines to ensure that food for human consumption is safe and has been prepared, packed, or held under sanitary condition to meet regulatory expectations and reduce the risk of product adulteration and food safety risk to consumers *(95)*. By using flexible terminology such as "adequate facilities", "where appropriate", "necessary precautions", and "adequate controls", this allows cGMPs to be applied to diverse situations during production, handling, and distribution of food products *(135)*.

3.3.3 Hazard Analysis and Critical Control Points (HACCP)

HACCP is a preventive system for assuring the safe production of food products and is based on application of technical and scientific principles to control chemical, physical, and biological hazards *(135)*. It was first developed by the Pillsbury Co. to assure the safety level of foods consumed by astronauts for the National Aeronautics and Space Administration (NASA) during space flight *(135)*. The seven principles proposed by the National Advisory Committee on the Microbiological Criteria for Foods (NACMCF) in 1997 are as follows *(85)*:

Principle 1: Conduct a hazard analysis;

Principle 2: Determine the critical control points (CCPs);

Principle 3: Establish critical limits;

Principle 4: Establish monitoring procedures;

Principle 5: Establish corrective actions;

Principle 6: Establish verification procedures;

Principle 7: Establish record-keeping and documentation procedures.

HACCP is designed to be used in all segments of the food industry from, harvesting, processing, manufacturing, distributing to preparing for consumption *(85)*. It cannot be implemented without prerequisite programs such as cGMPs and SSOPs *(85)*.

CHAPTER IV

PATHOGEN INTERVENTIONS FOR PRODUCE DECONTAMINATION AND SANITIZATION

Antimicrobial agents are commonly used in wash water to reduce microbial loads or to prevent cross-contamination of the products *(148)*. Generally, the efficiency of antimicrobials is affected by concentration, exposure time, temperature, pH, organic matter, number of microbial loads, and type of antimicrobials *(148)*. Ideal antimicrobial agents are expected to be: 1) able to destroy microorganisms rapidly and carry residual microbial inhibition effect, 2) easy to prepare and measure, 3) water soluble and stable, 4) tolerant to hard water, 5) environmentally compatible and non-toxic, 6) non-corrosive to containers and equipment utilized, 7) color and odor acceptable, and 8) economical *(148)*. Commonly used antimicrobials for disinfecting produce can include chlorine, chlorine dioxide, acidified sodium chlorite, and peroxyacetic acid *(90)*. Characteristics and research findings for each antimicrobial agent are discussed below.

4.1 Chlorine

Chlorine has been the most widely used sanitizer in the food agricultural and food industry *(90)*. When chlorine is added as a gas in water, it forms a mixture of hypochlorous acid (HOCl), which a is a strong oxidizing agent, and hydrochloric acid (HCl) *(59)*.

$$Cl_2 + H_2O \leftrightarrow HOCl + HCl$$

When Cl_2 exists in small amounts in the solution, the HOCl formed can dissociate as follows *(59)*:

$$HOCl \leftrightarrow H^+ + OCl^-$$

HOCl is the form of free available chlorine that possesses the highest bactericidal activity against a wide range of microorganisms *(90)*. The possible bactericidal mechanisms of chlorine include interference with microbial membrane functions, altered outer membrane permeability resulting in leakage of cell components, damage of proteins and enzymes, and nucleic acid destruction *(220)*. The amount of HOCl and hypochlorite (OCl^-) is pH dependent; the proportion of HOCl and OCl^- are equal at pH 7.9 at 0 °C *(148)*. As pH decreases, the amount of HOCL increases *(59)*. Nevertheless, at low pH, the solution becomes corrosive to equipment used and also cause off-gas *(148)*. Therefore, to minimize corrosion and maintain efficacy of chlorine, the solution pH should be adjusted to the range of 6.0 to 7.5 *(59)*. HOCl concentration also depends on temperature, presence or organic matter, light, air, and metal *(90)*. Chlorine can bind with organic substances in wash water, resulting in lower antimicrobial efficacy and formation of carcinogenic compounds such as trihalomethane and chloramine *(15, 265)*. In produce industry, liquid chlorine and hypochlorites are normally used at the concentration of 50 to 200 ppm with a contact time of 1 to 2 min *(90)*.

Zhang and Farber *(264)* reported reductions of *L. monocytogenes* at 4 and 22 °C were 1.3 and 1.7 $\log_{10}$ CFU/g for lettuce, and 0.9 and 1.2 $\log_{10}$ CFU/g for cabbage, respectively. Chlorine was also tested in combination with surfactants (Orenco Peel 40 and Tergitol); however, improved antimicrobial efficacy of chlorine was not observed *(264)*. A study by Wei et al. *(246)* showed 2 min dipping in 100 ppm chlorine could not effectively reduce (1.3 $\log_{10}$ reduction) *S.* Montevideo that was able to survive/grow in

tomato stem scars but could reduce the pathogen on tomato skin by 5.9 $\log_{10}$ CFU/g. Erkman *(82)* reported that 10 ppm of HOCL (pH 7.0) applied via immersion with agitation for 5 min reduced *E. coli* on lettuce, parsley and pepper by 1.23, 1.61, and 2.64 $\log_{10}$ CFU/ml respectively.

4.2 Chlorine Dioxide

Chlorine dioxide (ClO_2) is a synthetic water-soluble yellowish-green gas that has an odor similar to chlorine *(148)*. It has been used commercially as an alternative to chlorine for disinfecting fresh produce *(111)*. According to the FDA, ClO_2 in a concentration not to exceed 3.0 ppm can be used as an antimicrobial agent in water for washing fruits and vegetables that are not raw agricultural commodities *(96)*. Produce treated with ClO_2 must be followed by potable water rinse, blanching, cooking or canning *(96)*. ClO_2 is superior to free chlorine due to reduced reactivity with organic matter and greater activity at neutral pH *(114)*. Its oxidizing power is reported to be 2.5 times more effective than chlorine *(90)*. Since ClO_2 is explosive, it cannot be stored under pressure and cannot be shipped as gas; thus it has to be generated on-site *(114)*. The mechanism of action mainly involves non-specific oxidative damage of the outer membrane resulting in the destruction of the transmembrane ionic gradient *(114)*.

Han et al. *(121)* studied the effect of ClO_2 gas on survival of *E. coli* O157:H7 on injured green bell pepper surfaces. ClO_2 gas treatment for 30 min at 22 °C and 90-95% relative humidity yielded 6.5±0.02 $\log_{10}$ reduction *(121)*. Du et al. *(77)* found that ClO_2 gas treatment at a concentration of 4.0 ppm and a treatment time of 30 min inactivated *L. monocytogenes* attached to pulp skin (6.5±0.1 $\log_{10}$ reduction) more effectively than

those attached to calyx (4.3±0.2 $\log_{10}$ reduction) or stem cavity (4.3±1.1 $\log_{10}$ reduction). In another study, ClO_2 gas at 3 ppm and 0.6 ppm treatments inactivated more than 6.0 $\log_{10}$ CFU/5 g of *L. monocytogenes* on uninjured surfaces of green peppers and about 3.5 log CFU/5 g on injured surfaces respectively. The 3 ppm aqueous ClO_2 treatment yielded 3.7 and 0.4 $\log_{10}$ reductions on uninjured and injured green pepper surfaces respectively. Results suggested that ClO_2 gas treatment was more effective in reducing *L. monocytoge*nes on both uninjured and injured green pepper surfaces *(120)*.

4.3 Acidified Sodium Chlorite

Acidified sodium chlorite is generated by mixing an aqueous solution of sodium chlorite ($NaClO_2$) with any generally recognized as safe (GRAS) acid *(98)*. Acidified sodium chlorite can be used as an antimicrobial agent on raw agricultural commodities in preparing, packing, or holding of the food for commercial purposes *(98)*. If applied as a dip or spray, the concentration must be in the range of 500 to 1200 ppm, in combination with GRAS acid to obtain a final pH of 2.4 to 2.9 *(98)*. Potable rinse, blanching, cooking or canning must follow treatment of raw agricultural commodities with acidified sodium chlorite *(98)*. The antimicrobial activity of acidified sodium chlorite is due to the generation of chlorous acid ($HClO_2$) that possesses a strong oxidizing capacity *(148)*.

Allende et al. *(6)* reported that washing fresh cilantro with 1 g/L acidified sodium chlorite reduced *E. coli* O157:H7, mesophilic bacteria, and yeasts and molds by more than 3.0 $\log_{10}$ CFU/g. Also, reduction of *E. coli* O157:H7, mesophilic bacteria, and yeasts and molds by more than 2 $\log_{10}$ CFU/g were obtained after treatment with 0.25

and 0.5 g/L of acidified sodium chlorite. Park and Beuchat *(184)* reported 2.6 to 3.8 $\log_{10}$ reduction of *Salmonella* and *E. coli* O157:H7 populations inoculated onto surfaces of cantaloupes and honeydew melons after treatment with 1200 ppm sodium chlorite.

4.4 Peroxyacetic Acid

Peroxyacetic acid (or peracetic acid; PAA) is a strong oxidant disinfectant which contains an aqueous quaternary equilibrium mixture of acetic acid and hydrogen peroxide *(115)*. It can be produced from the reaction of acetic acid or acetic anhydride with hydrogen peroxide in the presence of sulfuric acid as a catalyst *(115)*. Peroxyacetic acid is a bright, colorless liquid with a strong pungent odor and pH approximately 2.8 *(148)*. Peroxyacetic acid is approved by the FDA to be used in wash water for fruits and vegetables that are not raw agricultural commodities at a concentration not to exceed 80 ppm *(97)*. The antimicrobial mechanism of peroxyacetic acid is mainly based on the release of reactive oxygen that can oxidize sensitive sulfhydryl and disulfide bonds in proteins, enzymes, and other metabolites of microorganism. This results in disruption of chemiosmotic functions (e.g. proton transfer) of the lipoprotein cell membrane and rupture of cell walls *(115)*.

Weller et al. *(249)* studied the effect of peroxyacetic acid on in-shell hazelnuts inoculated with *Salmonella* Panama. The spray treatment of 80 and 120 ppm of peroxyacetic acid reduced *S.* Panama populations by 1.3 and 1.5 log CFU/hazelnut *(250)*. Neo et al. *(171)* conducted a study to evaluate the effect of peroxyacetic acid on inactivating natural microbiota, acid-adapted and non-acid-adapted *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp. on mung beans. Immersion in 70 ppm PAA for 180

s resulted in 2.3, 1.8, 2.1 and 1.1 $\log_{10}$ reduction for non-adapted *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* spp., and natural microbiota, respectively. For acid-adapted microorganisms, $\log_{10}$ reductions in the range of 1.0 to 1.1 $\log_{10}$ CFU/g were obtained after the treatment.

CHAPTER V

ESCHERICHIA COLI O157:H7 AND *SALMONELLA ENTERICA*

5.1 *Escherichia coli*

5.1.1 Introduction

In 1885, Theodor Escherich, a German pediatrician, discovered an intestinal bacterium in feces of neonates and infants and named it "*Bacterium coli commune*"*(118)*. The bacterium was later renamed as *"Escherichia coli*" in his honor *(118)*. *E. coli* is the type species of the genus *Escherichia* that belongs to the family Enterobacteriaceae *(169)*. *E. coli* is a facultatively anaerobic commensal microbiota in intestinal tracts of humans and warm-blooded animals *(134)*. The commensal *E. coli* is normally harmless but can cause disease in debilitated or immunocompromised hosts *(134)*. Several strains, nevertheless, have acquired specific virulence factors that enable them to cause a wide range of diseases including diarrheal, urinary tract infections, sepsis, and meningitis *(134)*. *E. coli* can be serologically differentiated based on three major surface antigens including O (somatic), H (flagellar), and K (capsule) *(166)*. Normally, the O antigen identifies the serogroup and the H antigen identifies the serotype of a strain *(166)*.

5.1.2 *Escherichia coli* O157:H7

The uptake of mobile genetic materials (e.g. phages, virulence plasmids and pathogenicity islands) and the loss of chromosomal DNA play an important role in the evolution of *E. coli* into different pathogroups that cause specific symptoms in humans (Figure 3-1) *(4)*. Diarrheagenic *E. coli* can be classified into specific pathogroups

according to virulence properties, pathogenicity mechanisms, clinical syndromes, and specific O:H serotypes *(166)*. These include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and the diffuse-adhering *E. coli* (DAEC) *(134, 169)*.

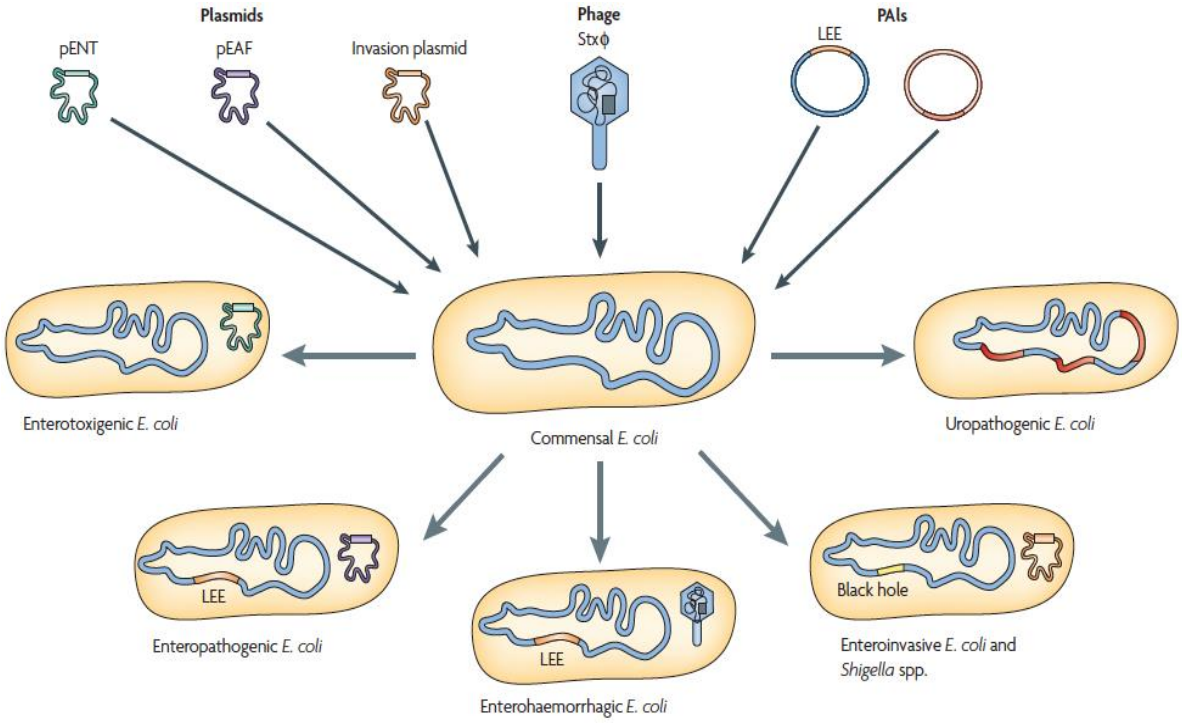

FIGURE 5-1. Evolution of *Escherichia coli* pathotypes from horizontal acquisition of mobile genetic elements. LEE, locus of enterocyte effacement; PAI, pathogenicity island; pEAF, enteropathogenic *E. coli* adhesion-factor plasmid; pENT, enterotoxin-encoding plasmids; Stx, Shiga-toxin-encoding bacteriophage *(4)*.

*E. co*li O157:H7 is a facultatively anaerobic rod-shaped, non-sporulating Gram-negative bacterium belonging to family Enterobacteriaceae *(127)*. Most strains of *E. coli* O157:H7 are unable to ferment the sugar alcohol sorbitol within 24 hr *(127)*. Also, the majority of *E. coli* O157:H7 are unable to grow well at temperature ≥44.5 °C in *E. coli* (EC) broth and are unable to produce β-glucuronidase *(166)*. Table 5-1 summarizes biochemical characteristics of most *E. coli* O157:H7. With an optimal growth temperature of 37°C, *E. coli* O157:H7 can grow at temperatures ranging from 8 to 42°C *(34, 134, 253)*. It can also grow at minimum pH and water activity of 4.4 and 0.95, respectively *(253)*.

TABLE 5-1. Summary of biochemical characteristics of most *E. coli* O157:H7 strains.

Biochemical test	**Reaction**
β-Glucuronidase	−
Sorbitol	−
Salicin	−
Esculin	−
Arginine dihydrolase	−
Adonitol	−
Inositol	−
Cellobiose	−
Urease	−
Citrate	−
KCN	−
Glucose (acid)	+
Indole	+
Arabinose	+
Trehalose	+
Mannitol	+
Lactose	+
Rhamnose	+
Xylose	+

TABLE 5-1. Continued.

Biochemical test	Reaction
Ornithine decarboxylase	+
Raffinose	+
Dulcitol	+

From *(192)*

E. coli O157:H7 is recognized as an enterohemorrhagic *E. coli* (EHEC) possessing a 60 MDa plasmid and causes diarrhea, hemolytic uremic syndrome (HUS), and hemorrhagic colitis *(166)*. The infectious dose of *S. enterica* can be 10 to 1,000 cells *(91)*. The pathogenesis of *E. coli* O157:H7 is associated with the ability to adhere to the host cell membrane and colonize the large intestine, followed by production of one or more Shiga-like toxins *(84, 141, 166, 175)* (Figure 5-5). *E. coli* O157:H7 interacts with host intestinal epithelial cells via the long protein filament (Lpf) fimbriae followed by formation of a histopathological feature known as an attaching-and-effacing (A/E) lesion. During A/E lesion formation, *E. coli* O157:H7 secretes effector proteins such as translocated intimin receptor (Tir), *E. coli* secreted protein F (EspF), *E. coli* secreted protein G (EspG), mitogen-activated protein (Map), etc. into the host cytoplasm through a type III secretion system (TTSS)*(166)*. Tir inserts into the host plasma membrane and binds to intimin, resulting in the formation of actin-rich pedestals beneath the attachment site after Wiskott-Aldrich syndrome protein (WASP) and recruitment of the heptameric actin-related protein Arp2/3 to the pedestal tip *(64, 84, 166, 172)*. Proteins involved in the formation of A/E lesion are encoded on a chromosomal pathogenicity island called the locus of enterocyte effacement (LEE) *(84, 152)*. *E. coli* O157:H7 produces Shiga-

like toxin 1 (Stx1) and Shiga-like toxin 2 (Stx2) *(134).* The Shiga-like toxins are encoded by genes carried by like prophages that are integrated into the chromosome of the *E. coli* O157:H7 hosts *(197).* Stx1 and Stx2 are 70 kDa proteins composed of one A subunit (32 kDa) and five B subunits (7.7 kDa) *(127, 225).* The A subunit contains an RNA N-glycosidase that cleaves the adenosine position of 28S ribosome of 60S ribosomal subunit. Cleavage of a single adenine nucleotide inhibits the elongation factor-1-dependent binding of the aminoaocyl-bound transfer RNA to the ribosome resulting in inhibition of protein translation and apoptosis *(166, 225).* The B subunits are responsible for tissue specificity by binding to globotriaosylceramide (Gb_3) or globotetraosylceramide (Gb_4) receptors on endothelial tissues of eukaryotes *(175, 225).* After binding to the glycolipid receptors, the toxin is internalized from clathrin-coated pits, transported to the trans-Golgi network (TGN), and then to the endoplasmic reticulum (ER) and the nuclear envelope respectively *(175).* In human, Gb_3 receptors are abundantly expressed on renal glomerular endothelial cells. Therefore, Shiga toxin production results in acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia which are all typical characteristics of HUS *(64, 172).* According to epidemiologic data, Stx2 seems to play a more important role in hemorrhagic colitis and HUS than Stx1 *(75, 116, 134, 141).*

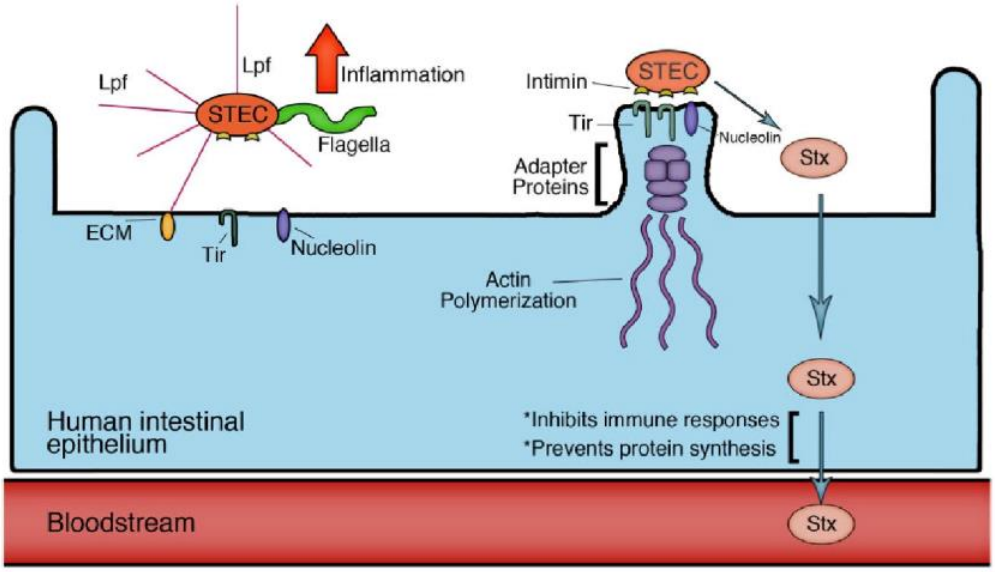

FIGURE 5-2. The first stage of colonization by *Escherichia coli* O157:H7 on human intestinal epithelial cells *(84)*.

Cattles are considered the primary source of *E. coli* O157:H7 in the food chain *(27)*. Other environmental factors such as water, feed sources, and improper manure handling can also play an important role in disseminating *E. coli* O157:H7 *(166)*. In 1982, Riley et al. *(195)* investigated two outbreaks of an unusual gastrointestinal illness that affected 47 people in Oregon and Michigan. Infected patients developed severe abdominal pain, initially watery diarrhea followed by grossly bloody diarrhea, and little or no fever *(195)*. The outbreak cause was believed to be consumption of undercooked hamburgers from McDonalds restaurants in Oregon and Michigan *(195)*. In 1995, there was an outbreak of *E. coli* O157:H7 infections associated with leaf lettuce consumption *(2)*. Forty Montana residents were identified with laboratory-confirmed *E. coli* O157:H7 infection. Thirteen patients were hospitalized and one patient developed HUS. The study

showed that prior to illness, 70% of patients consumed leaf lettuce, which was traced to a local Montana farm and six farms in Washington. The lettuce was shipped under the same label. The exact cause of contamination of *E. coli* O157:H7 in the lettuce leaf was not known. However, the possible causes were believed to be: 1) the use of contaminated compost to fertilize lettuce, 2) application of irrigation water cross-contaminated from cattle feces present in the adjacent uphill pasture and from other animals (e.g. sheep, deer) kept on the farm, and 3) feces of sheep or deer kept on the farm that could directly contaminate lettuce *(2)*. A multistate outbreak of *E. coli* O157:H7 linked to consumption of fresh spinach occurred in October, 2006. The Centers for Disease Control and Prevention (CDC) (Atlanta, GA) reported 199 people from 26 states in the United States were infected with the outbreak strain of *E. coli* O157:H7. Among the infected people, 102 people were hospitalized, 31 people developed HUS and 3 deaths were recorded. From 13 packages of spinach supplied by patients in 10 states, *E. coli* O157:H7 was isolated. However, the source of contamination of the outbreak strain was not identified *(49)*. In 2012, a multistate outbreak of *E. coli* O157:H7 associated with consumption of romaine lettuce happened. The CDC reported 58 people being infected with the outbreak strain from 9 states. Thirty-three people were hospitalized, three people developed HUS, and no deaths were reported. According to investigation of the outbreak, ill persons affected by this outbreak were reported to purchase lettuce from the salad bar from a regional grocery store chain. Trace-back investigations by the FDA and state agencies indicated that a single common lot of romaine lettuce harvested from Farm A was used to supply the grocery store chain

locations and university campuses in Minnesota and Missouri during the time of the outbreak. Nevertheless, the source of contamination at Farm A could not be identified *(53)*.

5.2 *Salmonella enterica*

Salmonella is a facultatively anaerobic, rod shaped, non-sporulating Gram-negative bacterium belonging to family Enterobacteriaceae *(206)*. The genus *Salmonella* consists of two species, *S. bongori* and *S. enterica*. *S. enterica* can be classified into six subspecies including *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* *(242)*. The O and H antigens can be used to differentiate *Salmonella* serologically *(65)*. In humans and warm-blooded animals, approximately 99% of *Salmonella* infections are caused by *S. enterica* subsp. *enterica (242)*. Most strains of *S. enterica* are motile with peritrichous flagella *(206)*. However, the *S. enterica* subsp. *enterica* serovar Pullorum and *S. enterica* subsp. *enterica* serovar Gallinarum are non-flagellated *(65, 194)*. *S. enterica* is oxidase negative and catalase positive. Most *S. enterica* strains can utilize citrate as a sole carbon source, produce hydrogen sulfide, decarboxylate lysine and ornithine, but cannot hydrolyze urea *(65)*. Table 5-2 summarizes biochemical characteristics of most *S. enterica* strains. With an optimal growth temperature of 37 °C, *S. enterica* can grow at temperature between 5.3 and 45 °C *(132)*. *S. enterica* can grow at pH 4 to 9 with the optimal growth pH around 6.6 to 8.2 *(65, 132)*. Normally *S. enterica* cannot tolerate the presence of 3 to 4% NaCl; however, survival of *S. enterica* in 17% NaCl was reported *(10, 65, 132)*.

TABLE 5-2. Summary of biochemical characteristics of most *S. enterica* strains.

Biochemical test	Reaction
Glucose (Triple sugar iron agar)	+
Lysine decarboxylase (Lysine iron agar)	+
H_2S (Triple sugar iron agar and lysine iron agar)	+
Urease	–
Lysine decarboxylase broth	+
Phenol red dulcitol broth	+[(b)]
KCN broth	–
Malonate broth	–[(c)]
Indole test	–
Polyvalent flagellar test	+
Polyvalent somatic test	+
Phenol red lactose broth	–[(c)]
Phenol red sucrose broth	–
Voges-Proskauer test	–
Methyl red test	+
Simmon's citrate	v

[a] +: 90% or more positive in 1 or 2 days; -: 90% or more negative in 1 or 2 days; v: variable.
[b] Majority of *S. Arizonae strains* are negative.
[c] Majority of *S. Arizonae* strains are positive.
From *(94)*

S. enterica can cause human salmonellosis, which includes symptoms of gastroenteritis, enteric fever, and septicemia *(66)*. The infectious dose of *S. enterica* can be 10 to 100,000 cells *(91)*. Once ingested, *S. enterica* from food passes through the gastric acid barrier in the stomach and attaches to the small intestinal columnar epithelial cells and specialized microfold (M) cells using invasion appendages *(113, 124, 266)*. Then, *S. enterica* assembles the *Salmonella* pathogenicity island 1 (SPI1)-encoded type III secretion system and translocates invasive proteins *Salmonella* invasive protein A

(SipA), *Salmonella* invasive protein B (SipB), *Salmonella* invasive protein C (SipC), *Salmonella* outer protein E2 (SopE2), and *Salmonella* outer protein B (SopB) to eukaryotic cells *(61, 65)*. Activation of host Rho GTPases by invasive proteins results in the rearrangement of the actin cytoskeleton into membrane ruffles, induction of mitogen-activated protein kinase pathways, destabilization of tight junctions, and a Ca^{2+} influx in the host intestinal epithelial cells and M cells *(65, 124)*. Invasion of *S. enterica* in intestinal epithelial cells and M cells also results in stimulation of pro-inflammatory cytokine release that induces inflammatory reaction *(113)*. The inflammatory response causes diarrhea and may also result in ulceration and mucosa destruction *(113)*. Systemic infection can occur if the bacteria disseminate from the intestines *(113)*.

S. enterica has been identified as the leading cause of foodborne bacterial illness in humans *(65)*. Poultry is the main reservoir of *S. enterica (65)*. In 2006, an outbreak of *S.* Typhimurium infection caused by contaminated tomatoes at restaurants occurred in 21 states throughout the U.S. *(46)*. There were 183 infected patients *(46)*. Most of them exhibited fever and diarrhea; no deaths were reported. A multi-national outbreak of *S.* Saintpaul occurred in 43 states of the U.S., the District of Columbia and Canada in 2008 *(51)*. The CDC reported 1442 infected patients; most had developed diarrhea, fever and abdominal cramps, 12 to 72 hours after ingestion *(51)*. The information indicated that jalapeños and tomatoes grown, harvested or packed in Mexico during that time were the cause of this outbreak *(51)*. In 2013, a multistate *Salmonella* Saintpaul outbreak implicated in cucumbers occurred in 18 states *(55)*. There were 84 people infected with the outbreak strain; 23 people were hospitalized and no deaths were reported *(55)*. The

cause of the outbreaks were traced back to be consumption of imported cucumbers supplied by Daniel Cardenas Izabal and Miracle Greenhouse of Culiacan, Mexico and distributed by Ticar sales, Inc. of Rio Rico, Arizona *(55)*. Thirty-four of 49 infected persons reported consuming various types of cucumbers purchased from the grocers or restaurants distributed by Ticar Sales *(55)*. Importing cucumbers from the two supplier firms, Daniel Cardenas Izabal and Miracle Greenhouse of Culiacan, into the U.S. was later suspended until the supplier could prove that the products were no longer contaminated *(55)*.

CHAPTER VI

ESSENTIAL OIL COMPONENTS

6.1 Introduction

Essential oil components (EOCs) are naturally occurring aromatic volatile compounds in plants, composed of heterogeneous mixtures of organic compounds such as terpenes, phenolpropanes, alcohols, esters, ketones, aldehydes, acids and phenols *(247)*. Normally they can be obtained from differing plant tissues (e.g. buds, flowers, seed, leaves, twigs, fruits, bark, woods, and roots) by steam distillation or solvent extraction *(36, 209)*. EOCs have been reported to possess antimicrobial activities against a wide range of pathogens *(19, 228, 234, 236)*. Normally, the most potent antimicrobial activities have been reported in EOCs containing phenolic groups (e.g. eugenol, carvacrol, and thymol) *(199, 209, 247)*. Eugenol (4-allyl-2-methoxyphenol), carvacrol (5-isopropyl-2-methyl-phenol), and thymol (2-isopropyl-5-methylphenol) are EOCs from clove (*Syzygium aromaticum*), oregano (*Origanum vulgare*), and thyme (*Thymus vulgaris*), respectively (Figure 6-1) *(67)*. They have been reported to exhibit antimicrobial activity against several foodborne pathogens *(15, 67)*. Kim et al. *(144)* reported minimal inhibitory concentrations (MICs) of eugenol against *S.* Typhimurium and *L. monocytogenes* were 1.0 and 0.5 µl/ml, respectively. Cosentino et al. *(63)* reported MICs of thymol against *E. coli*, *S.* Typhimurium. *S. aureus*, *L. monocytogenes* and *B. cereus* incubated aerobically at 37°C for 24 h ranged from 0.056 to 1.0 µl/ml. Due to the generally recognized as safe (GRAS) status of EOCs, they can be used as food additives for human consumption *(95)*.

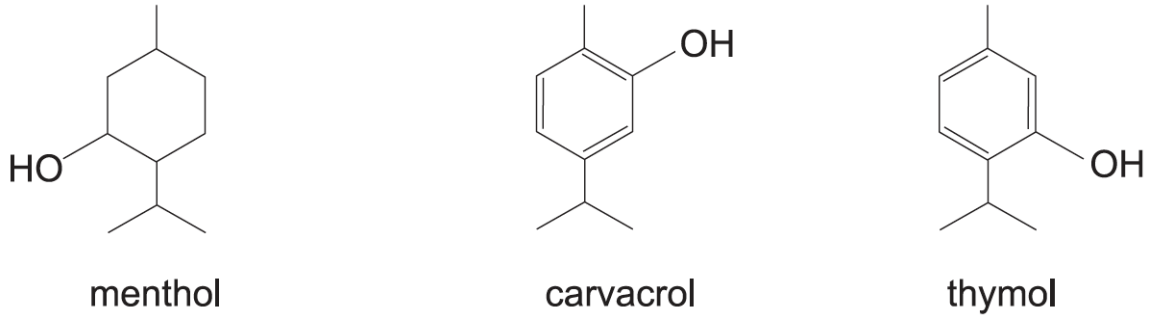

FIGURE 6-1. Structures of selected essential oil components *(36)*.

6.2 Mechanisms of Action of Essential Oil Components

The possible mechanisms of action of EOCs against microorganisms include cytoplasmic membrane disruption, destabilization of proton motive force (PMF), electron flow, active transport, and coagulation of the cell content, reduction of ATP production, pH disturbance, intra-cytoplasmic changes, DNA mutation, and disturbance of quorum sensing (Figure 6-2) *(83, 146, 212)*. Bouhdid et al. *(29)* studied cellular effect of *Origanum compactum* essential oil on *P. aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 using plate count, potassium leakage, flow cytometry, and transmission electron microscopy (TEM). Results showed that treatment of *P. aeruginosa* and *S. aureus* with the EOC caused reduction of cell viability and dissipated K^+ gradients, and loss of membrane potential and permeability. TEM results showed coagulated cytoplasmic constituent and liberation of membrane vesicle in EOC-treated *P. aeruginosa* and mesosome-like structures in EOC-treated *S. aureus.* Flow cytometry and TEM results suggested that the EOC has a more significant effect in *P. aeruginosa* than in *S. aureus.* The differences in the effect of the EOC on the two

pathogens are mainly due to differences in the membrane and cell wall structure and composition.

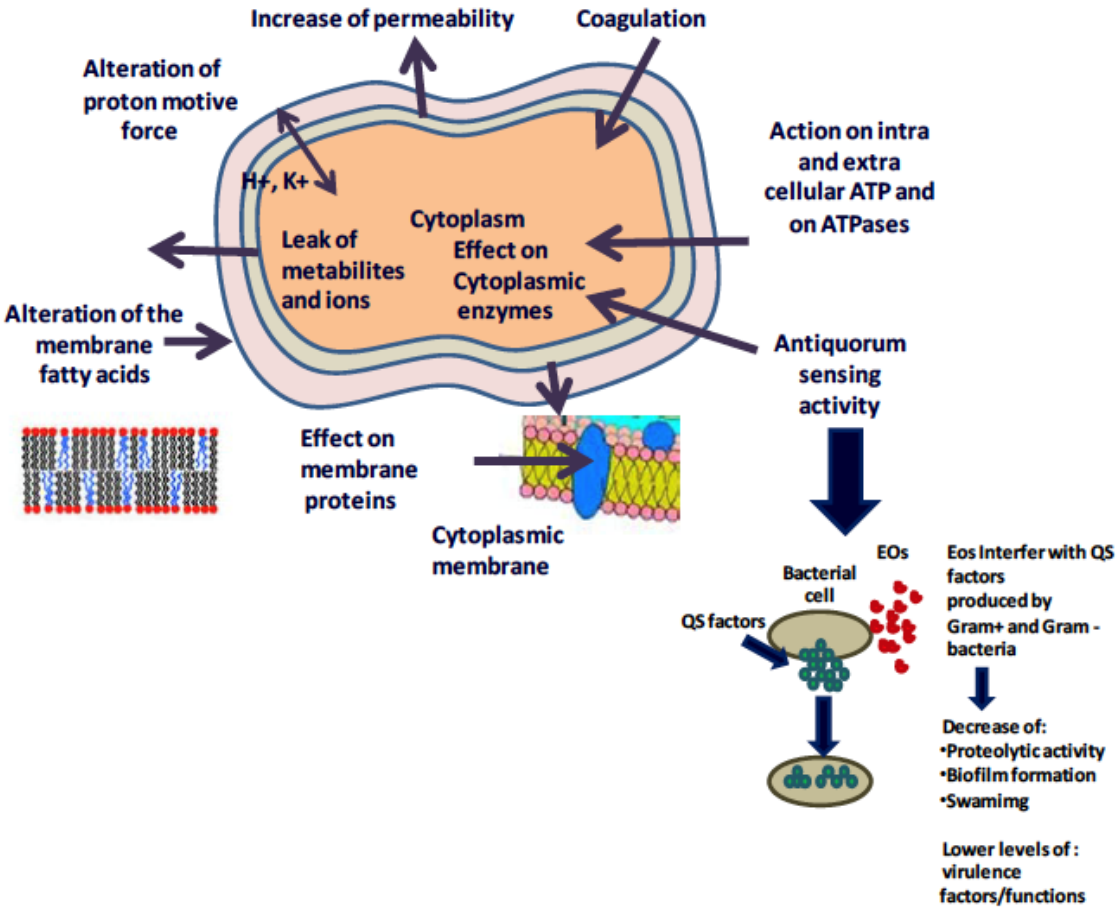

FIGURE 6-2. Possible mechanisms of action of essential oils at target sites of microorganisms *(170)*.

Szabo et al. *(221)* studied inhibitory effect of essential oils on quorum sensing signals between the sensor strain *Chromobacterium violaceum* CV026 and *N*-Acyl

homoserine lactone (AHL) producing *E. coli* ATCC 31298 and the grapevine colonizing Ezf 10-17 strains. At the concentration of 10% and 100%, geranium, lavender oil, and rosemary oil efficiently inhibited color production of *C. violaceum* CV026 to the AHL produced by *E. coli* ATCC 31298 and Ezf 10-17. Pigment production of the *C. violaceum* CV026 was moderately decreased by eucalyptus and citrus oils, but not affected by chamomile, orange and jumper oils *(221)*. Both hydrophilic and hydrophobic parts of EOCs are involved in antimicrobial activity *(209)*. For example, the hydrophilic parts (e.g. hydroxyl groups) of EOCs interact with polar parts (e.g. outer membrane proteins) of the bacterial cell membrane while the hydrophobic component(s) react with lipids on the membrane and result in increased membrane permeability, disturbed enzyme systems and genetic materials *(209, 211, 222)*.

EOCs have also been reported to inhibit bacterial spore germination *(149, 186, 210)*. Lawrence et al. *(149)* studied the effect of 13 EOCs including bergamot, cardamom, clove bud, eucalyptus blue gum, Jupiter leaf, laurel leaf, lemongrass, palmarosa, peppermint, pine, tea tree, thyme, and yarrow oil. Spores of *Bacillus subtilis* were exposed to 13 essential oils individually *(149)*. Cardamon, tea tree, and juniper leaf oil exhibited the most potent spore inhibitory effect, resulting in more than 3 $\log_{10}$ reduction at oil concentration above 1% *(149)*. Scanning electron microscopy (SEM) indicated that EOC treatment caused visible damage to the spore coat, suggesting that leakage of spore contents likely happened *(149)*. Voundi et al. *(243)* studied the effects of different types of 9 EOCs on spores of *Geobacillus stearothermophilus*, *Bacillus megaterium*, *B. cereus*, and *B. subtilis* solid medium (Muller-Hinton agar) and broth. In

broth assay, the EOC from *Drypetes gossweileri* (containing 86.7% benzyl isothiocyanate) was the most potent among all 9 EOCs, yielding MIC for inhibition of spore germination (MICg) of *B. megaterium*, *B. cereus*, and *B. subtilis* at 0.002, 0.002, and 0.001 mg/ml respectively (MICg for *G. stearothermophilus* was not determined) *(243)*. In solid medium, the EOC from *D. gossweileri* also exhibited the most effective inhibitory effect on spore germination among the 9 EOCs *(243)*. At 1.25 mg/ml, EOC from *D. gossweileri* reduced spore germination of tested microorganisms by 50% or more *(243)*.

Although EOCs contain a hydroxyl group that renders them partially hydrophilic, their inherent hydrophobicity has been suggested to lead to a requirement for higher concentrations of oils for foodborne pathogen inhibition due to reduced water solubility, or to use of technologies allowing EOC dispersion in aqueous phases of foods where microbial organisms reside, such as the application of micro-encapsulation in surfactant micelles *(110, 196, 247)*. Also, when EOCs are applied in food systems, higher concentrations might be required to inhibit foodborne pathogens due to low solubility in aqueous phases *(20, 42, 207, 247)*. The high concentration of EOCs might exceed the maximum allowance in foods and might also affect sensory attributes of foods *(110, 247)*.

CHAPTER VII

SURFACTANTS AND MICELLES

7.1 Surfactants

Surfactants are surface-active amphiphilic compounds that contain hydrophilic and hydrophobic groups *(104, 109)*. In aqueous solution, surfactants adsorb to the solution/air interface with the hydrophobic group protruding into the vapor phase to minimize exposure of hydrophobic groups to polar aqueous solution *(104)*. This results in decreased surface tension (the strength of liquid surface contraction expressed by the work required to increase the surface area by 1.0 m^2), since surfactant molecules replace certain water molecules at the aqueous surface and the attraction forces between surfactant molecules and water molecules are less than those between water molecules *(104)*. Surfactants can be categorized as non-ionic (e.g. Triton X, Tween 20) anionic (e.g. sodium dodecyl sulfate), cationic (e.g. lauric arginate ester), or zwitterionic (e.g. cocamidopropyl betaine*) (11, 104, 248)*.

7.2 Micelles

At low concentrations, surfactants exist as monomers in solution *(164)*. At or above a critical micelle concentration (CMC), surfactant monomers aggregate with each other and form thermodynamically driven colloidal structures such as micelles, bilayers and vesicles. A surfactant CMC can be affected by many factors including the chain length of the hydrocarbon (increased chain length decreases CMC of surfactants with identical polar head groups), nature of hydrophilic group (CMCs of nonionic surfactants are normally lower than ionic surfactants), counterion (ionic surfactants containing

organic counterions have lower CMCs), electrolytes (CMC decreases upon electrolyte addition), and temperature (CMC decreases at temperature up to the cloud point) *(74, 104)*. For micelles, the surfactant hydrophobic tail groups reside inside the micelle core and hydrophilic head groups protrude into the aqueous phase *(109, 160)*. The driving force of micelle formation is the hydrophobic effect, which causes the surfactant molecules to minimize unfavorable contact between the hydrophobic groups and the surrounding aqueous phase *(164)*. Generally, the shape of micelles at CMC is considered spherical or nearly spherical *(150)*. With increased surfactant concentrations, spherical micelles can change into rod-like, lamellar or vesicle-like micelles *(150)*. During storage, micelle size may also change due to flocculation (a process by which two or more droplets associate with each other to form an aggregate but individual integrities are still retained), coalescence (a process by which two or more droplets fuse together resulting in a single larger droplet), and Ostwald ripening (a process by which large droplets grow at the expense of smaller droplets) *(161, 163)*. The shape of micelles is largely determined by surfactant molecular geometry, surfactant chemical structure, solution composition and temperature *(164)*. The average size of empty micelles is 3 to 4 nm *(247)*. The size of micelles is determined by many factors such as increased hydrocarbon chain length, types of counter-ion, charge of the hydrophilic group, and addition of electrolytes *(12)*. Surfactants can be used to encapsulate hydrophobic compounds to improve their solubility in the aqueous phase *(109)*. The maximum amount of hydrophobic compounds that can be incorporated in micelles is called the maximum additive concentration (MAC) or solubilization capacity (SC) *(247, 248)*.

Upon incorporation of hydrophobic compounds, the size of micelles increases 3 to 4X their original size and reach the maximum size at MAC *(247)*. Factors affecting MACs of micelles can include the nature of surfactant (e.g. increased MAC of hydrophobic compound residing in the micelle core with increasing surfactant alkyl chain length up to C_{16}), nature of hydrophobic compounds (e.g. decreased MAC with increasing alkyl chain length), temperature, and the hydrophile-lipophile balance (HLB) of surfactant *(12, 107, 248)*. The three possible mechanisms of solubilization of hydrophobic compounds (e.g., oil) by surfactants include: 1) direct oil solubilization in aqueous solution followed by incorporation into micelles in the aqueous phase; 2) uptake of oil by empty micelles due to collision of micelles with the surface of an emulsion droplet, and; 3) spontaneous "budding-off" of oil and surfactant molecules from a droplet surface to form micelles *(159)*. Solubilization is thermodynamically driven by reduction of the system Gibbs free energy by obtaining the optimal organization of micelles to minimize contact between hydrophobic compounds and the polar aqueous phase *(109)*.

7.3 Surfactant-based Antimicrobial Encapsulated-Nanoparticles for Inhibition of Foodborne Pathogens

Utilization of surfactant-based antimicrobial encapsulated-nanoparticles has been reported in previous studies. Gaysinsky et al. *(107)* reported that eugenol encapsulated in Surfynol 485W micelles was the most efficient in inhibiting growth of foodborne pathogens. The results showed 0.15% eugenol encapsulated in 1% Surfynol 485W inhibited growth (7.0 $\log_{10}$ CFU/ml inocula) of three *L. monocytogenes* strains and four *E. coli* O157:H7 strains in tryptic soy broth *(107)*. In skim milk, 5% Surfynol 485W with

0.5% eugenol completely inhibited growth (4.0 $\log_{10}$ CFU/ml inoculums) of two strains of *L. monocytogenes* for up to 48 hr and reduced numbers of two strains of *E. coli* O157:H7 to undetectable levels in less than 1 h *(110)*. Chang et al. *(60)* studied antimicrobial activities of carvacrol nano-emulsions consisting of carvacrol, 10% medium chain triglyceride, and 10% Tween 80 against *Zygosaccharomyces bailii*, *Saccharomyces cerevisiae*, *Brettanomyces bruxellensis*, and *Brettanomyces naardenensis* (4.0 $\log_{10}$ CFU/ml) in malt extract (ME) broth. The lowest MIC (158 µg/ml carvacrol) was observed against *B. naardenensis (60)*. Pérez-Conesa et al. *(70)* reported 5% Surfynol 485W micelles containing 0.7% carvacrol and 0.9% eugenol inactivated biofilms of two strains of *E. coli* O157:H7 grown on stainless steel coupons by 3.5 to 4.8 $\log_{10}$ CFU/cm^2 within 20 min. In another study by these authors, biofilms of four strains of *E. coli* O157:H7 and *L. monocytogenes* grown on polycarbonate membranes were treated with 0.3 to 0.9% of carvacrol and eugenol encapsulated in 5% Surfynol 485W *(189)*. Colony biofilms of all *E. coli* O157:H7 strains and three *L. monocytogenes* strains were inactivated to undetectable levels after exposure to the EOC-bearing micelles for more than 3 h *(189)*. Ma et al. *(22)* studied antimicrobial properties of lauric arginate ester (LAE) alone or in combination with thymol, eugenol and cinnamon oil in tryptic soy broth and 2% reduced fat milk. Cinnamon oil or eugenol in LAE micelles showed synergistic effect (fractional inhibitory concentration index; FIC_I <1) against *L. monocytogenes* while thymol in LAE micelles showed additive effect (FIC_I = 1). Antagonistic effects (FIC_I <1) of thymol, eugenol and cinnamon oil in LAE micelles against *E. coli* O157:H7 and *S.* Enteritidis were observed *(22)*. Were et al. studied

antimicrobial activities of nisin and lysozyme encapsulated in phosphatidylcholine (PC), PC-cholesterol, and PC-phosphatidylglycerol (PG)-cholesterol liposome against five strains of *L. monocytogenes (251)*. In tryptic soy broth (TSB), nisin encapsulated in PC and PC-cholesterol liposomes reduced growth of *L. monocytogenes* by greater than 2.0 $\log_{10}$ CFU/ml compared to free nisin, which reduced growth by less than 2.0 $\log_{10}$ CFU/ml *(251)*. Lysozyme encapsulated in PC-cholesterol and PC-PG-cholesterol liposome inhibited one strain of *L. monocytogenes* by more than 2.0 $\log_{10}$ CFU/ml *(251)*. Antimicrobial activity against *E. coli* O157:H7 and *L. monocytogenes* of nisin (5.0 and 10.0 g/ml) and ethylene diaminetetraacetic acid (EDTA) encapsulated in phospholipid liposome was studied by Taylor et al. *(223)*. From the study, phosphatidylcholine (PC) liposomes did not show significant inhibition against target pathogens while PC:phosphatidylglycerol 8:2 and 6:2 (mol%) exhibited inhibition of pathogens *(223)*. These studies suggested that surfactant-based antimicrobial encapsulated-nanoparticles could provide effective activities against foodborne pathogens and might be a promising method for food system application.

CHAPTER VIII

ENUMERATION OF NATIVE MICROBIOTA ON FRESH PRODUCE

8.1 Materials and Methods

8.1.1 Preliminary Study for Tomato Sampling Procedures

A preliminary study was conducted to determine: 1) the inhibitory effect on nativemicrobiota due to sampling procedures and 2) the ability of native microbiota to grow in diluent solutions within 1 h prior to plating. Roma tomatoes were obtained from a local grocer (College Station, TX). Three different sampling procedures (coring, skin excision, and sponge swabbing) were used for tomato sampling. Coring was performed using a flame-sterilized stainless steel cork borer to sample three pieces (10 cm^2 each) of tomato with flesh attached to the skin. Skin excision was performed using a flame-sterilized cork borer and scalpel to excise the skin of tomato samples without flesh attached to the skin. For sponge swabbing, a sponge was used to swab a surface of 30 cm^2 of tomato samples. Sampled tomato pieces and sponge were placed in a stomacher bag. Phosphate-buffered saline (PBS; Thermo-fisher Scientific, Waltham, MA), which provided acid neutralization capacity, or 0.1% peptone water diluent (PW; Becton, Dickinson and Co., Sparks, MD) was added to the bag (100 ml for coring and excision procedure; 25 ml for sponge swabbing procedure). Samples were pummeled using a stomacher (bioMérieux N.A., Durham, NC) (230 rpm; for coring and excision procedure) or hand stomaching (for sponge swabbing procedure) and were held for 0, 0.5 and 1 h prior to plating on Petrifilm™ Aerobic Count Plates (3M; Maplewood, MN). All samples were further serially diluted in 9.0 ml PW to obtain serial dilutions.

Petrifilms were incubated at 35 °C for 24 h prior to enumeration. The experiment was performed in triplicate (n=3).

8.1.2 Enumeration of Native Microbiota on Fresh Produce Commodities

Lettuce, spinach, and parsley (n=6 per farm) were obtained in spring harvest seasons from differing produce growing operations in South Texas. Jalapeño pepper, tomato, and cantaloupe (n=6 per farm; n=12 per season) were obtained in spring and fall harvest seasons from differing produce growing operations in south Texas. Twenty-five gram samples of leafy green commodities (lettuce, spinach, and parsley) were pummeled in 225 ml of 0.1% (w/v) PW for 1 min. Three 10 cm^2 samples of pepper and cantaloupe were aseptically excised from the surfaces using a sterile scalpel and then pummeled in 100 ml of 0.1% (PW). Three 10 cm^2 samples of tomato excisions were pummeled in 100 ml PBS. Stem scar areas of cantaloupe and tomato were measured using a sterile caliper and were aseptically excised using a sterile scalpel; stem scar samples were vortexed in 10 ml PW for 1 min. All samples were further serially diluted in 9.0 ml PW to obtain serial dilutions and spread on TSA, *Pseudomonas* agar F (Becton, Dickinson and Co.), *Pseudomonas* agar P (Becton, Dickinson and Co.), Kenner Fecal (KF) Streptococcal agar, (Becton, Dickinson and Co.) and Violet Red Bile (VRB; Becton, Dickinson and Co.) Agar supplemented with 0.01% (w/v) 4-methylumbelliferyl-β-D-glucuronide (MUG; Sigma Aldrich Co., St. Louis, MO) to enumerate aerobic bacteria, *Pseudomonas* with fluorescein production ability, *Pseudomonas* with pyocyanin production capacity, total *Enterococcus* spp., total coliforms and *Escherichia coli,* respectively. Inoculated

Petri dishes containing media were incubated at 35 °C for 24 h. DeMan, Rogosa, and Sharpe (MRS; Becton, Dickinson and Co.) and All Purpose Tween agar (APT; Becton, Dickinson and Co.) Lactobacilli agar were used to detect homofermentative and heterofermentative Lactic Acid Bacteria (LAB), respectively, and were incubated anaerobically at 35°C for 48 h. Yeast and mold enumeration was completed using Dichloran Rose Bengal Chloramphenicol agar (DRBC; Becton, Dickinson and Co.) with incubation at 27 °C for 5 days. Colonies were enumerated after incubation *(16, 26, 32, 125, 145, 168).* The microbiological data obtained from the assay were subject to statistical analysis to determine differences amongst microbial counts from differing microbial groups between harvest seasons.

8.1.3 Statistical Analyses

Prior to statistical analysis, microbial count data were logarithmically transformed (base 10). Statistical analyses of the logarithmically-transformed data were performed using JMP v10.0.0 (SAS Institute Inc., Cary, N.C.). Significant differences ($p < 0.05$) amongst mean $\log_{10}$ counts were determined by analysis of variance ANOVA and differing means were separated using Student's t-test.

8.2 Results and Discussion

8.2.1 Preliminary Study on Tomato Sampling Procedures

Figure 8-1 shows the aerobic plate counts from different sampling procedures, diluents and holding periods. The counts ranged from 1.9±0.6 to 2.6±1.4 $\log_{10}$ CFU/cm^2 and were not statistically different ($p \geq 0.05$). Furthermore, the effect of sampling procedures ($p \geq 0.05$), diluents ($p \geq 0.05$), and holding time prior to plating ($p \geq 0.05$) did not impact aerobic bacterial counts. Thus, tomato flesh acidity from coring procedure did not adversely affect microbial counts of samples. Also, holding the pulverized samples up to 1 h prior to plating did not impact the counts (Fig. 8-1). Although statistical differences were not observed, skin excision with PBS diluent seemed to produce the lowest variability among other procedures and the pH of PBS was also close to neutrality. Thus, skin excision with PBS as a diluent was chosen for sampling tomato native microbiota in section 8.1.2.

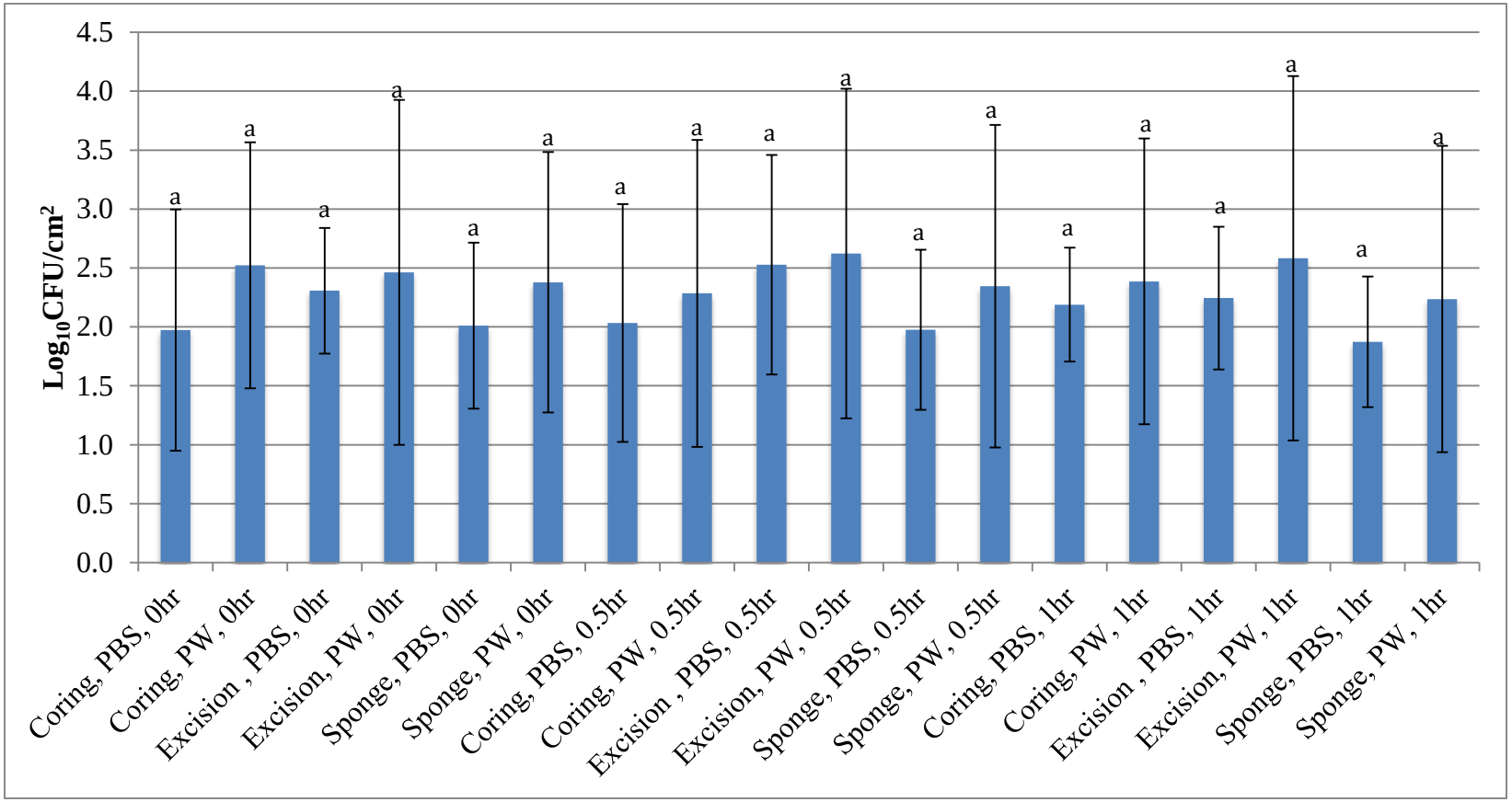

FIGURE 8-1. Aerobic plate counts from tomato skins using different sampling procedures (coring, excision, and sponge swabbing), diluent (PBS, and PW), and holding time prior to plating (0 hr, 0.5 hr, and 1 hr). The columns depict means from triplicate identical replications (n = 3) while error bars depict standard deviation from the mean. Columns not connected by the same letter are statistically different ($p<0.05$). Limit of detection is 0.5 $\log_{10}$ CFU/cm^2.

8.2.2 Native Microbiota on Leafy Greens

8.2.2.1 Native Microbiota on Lettuce

For farm 1 (n=6, Temp=24 °C, ERH=81% at day of harvest), the populations of aerobic bacteria, pseudomonads from PF agar, fluorescein-producing pseudomonads, pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB were 6.0±0.2, 6.2±0.1, 1.3±1.5, 5.9±0.1, 0.7±0.0, 4.5±0.2, 2.3±1.0, 4.7±0.8, 1.7±1.2, 5.2±0.2, 6.1±0.2 $\log_{10}$ CFU/g respectively (Figure 8-2). For farm 2 (n=6, Temp=16 °C, ERH=51%), the populations of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB were 6.1±0.2, 5.8±0.2, 1.3±1.4, 5.8±0.2, 0.7±0.0, 5.4±0.2, 3.7±0.3, 5.0±0.5, 2.6±1.0, 3.8±0.4, 5.4±0.5 $\log_{10}$ CFU/g (Figure 8-2). Total pseudomonads (from PF agar), homofermentative and heterofermentative LAB populations on lettuce from farm 1 were higher than those from farm 2 ($p<0.05$). Yeasts and molds, and enterococci populations, from farm 2 were higher than those from farm 1 ($p<0.05$). The populations of aerobic bacteria, fluorescein-producing pseudomonads, total pseudomonads (from PP agar), pyocyanin-producing pseudomonads, total coliforms, and *E. coli* did not differ between both farms ($p\geq0.05$).

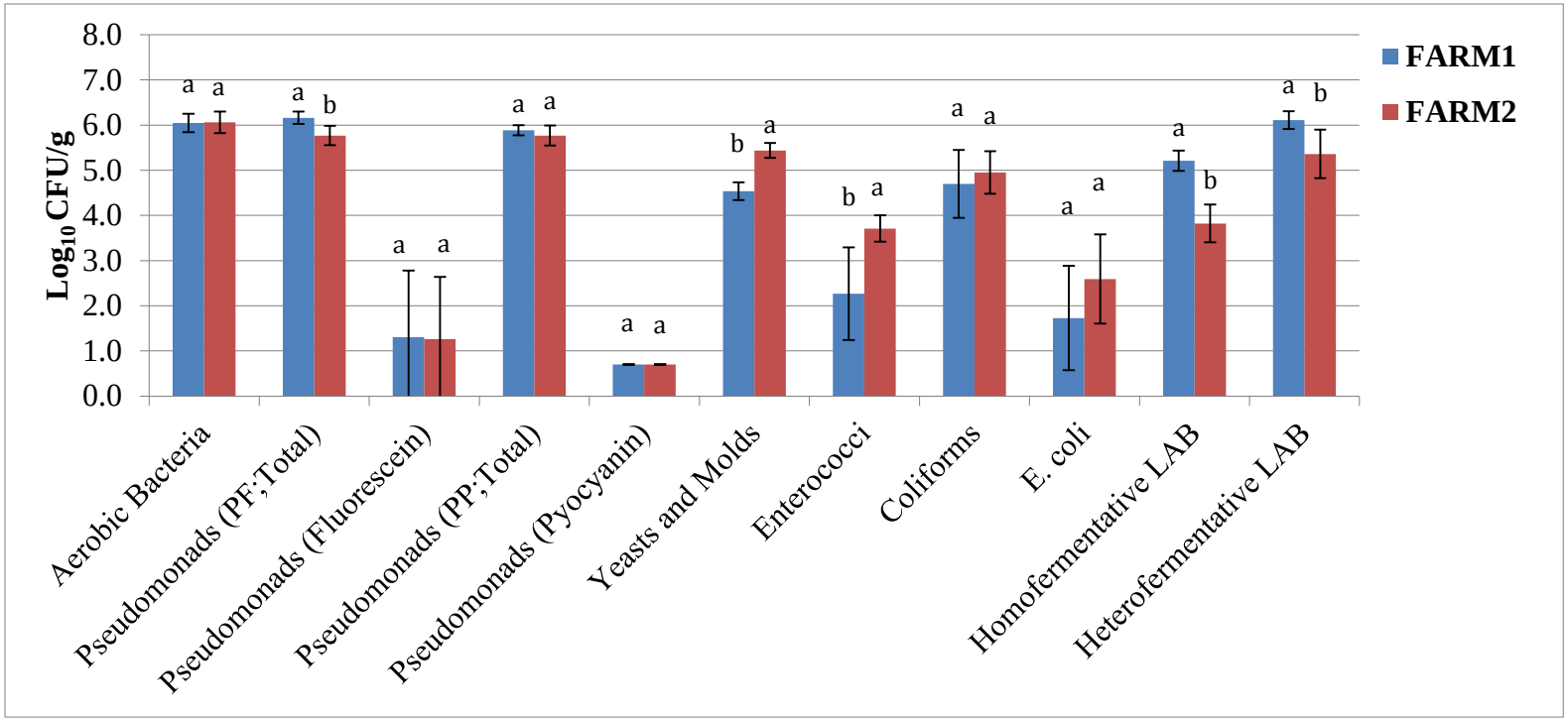

FIGURE 8-2. Log$_{10}$ CFU/g of native microbiota on surfaces of lettuce samples from two Rio Grande valley farms. Columns represent mean log$_{10}$ CFU/g of native microbiota; error bars indicate standard deviation from sample mean. Within microbial grouping, columns not sharing the same letter are statistically different ($p<0.05$). The limit of detection is 1.0 log$_{10}$ CFU/g.

8.2.2.2 Native Microbiota on Spinach

For farm 1 (n=6, Temp=18 °C, ERH=90%), the populations of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB were 6.5±0.4, 6.5±0.4, 2.6±2.2, 6.5±0.4, 0.7±0.0, 4.2±1.1, 2.8±0.7, 5.3±1.1, 1.6±1.0, 5.5±1.1, 6.6±0.5 $\log_{10}$ CFU/g respectively (Figure 8-3). For farm 2 (n=6, Temp=22 °C, ERH=81%), the populations of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB were 7.2±0.3, 7.0±0.3, 4.3±1.8, 6.9±0.3, 4.3±1.8, 5.5±0.3, 4.3±0.3, 5.7±0.7, 3.9±0.6, 5.6±0.6, 6.6±0.3 $\log_{10}$ CFU/g (Figure 8-3). Aerobic bacteria, total pseudomonads (from PF agar), pyocyanin-producing pseudomonads, yeasts and molds, enterococci, and *E. coli* populations from farm 2 were higher than those from farm 1 ($p<0.05$). The populations of fluorescein-producing pseudomonads, total pseudomonads (from PP agar), homofermentative LAB, and heterofermentative LAB were not different between both farms ($p\geq0.05$).

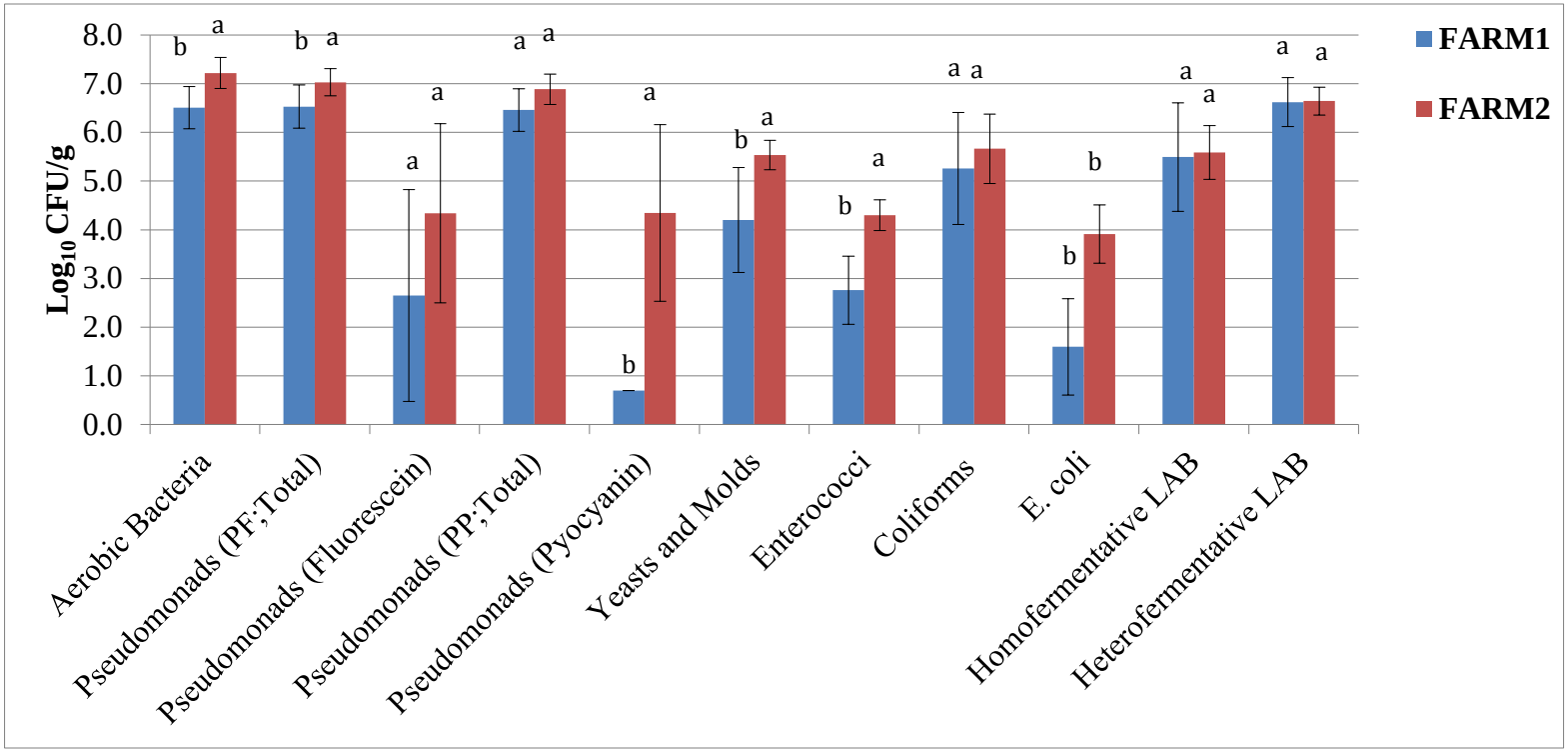

FIGURE 8-3. Log_{10} CFU/g of native microbiota on surfaces of spinach samples from two Rio Grande valley farms. Columns represent mean log_{10} CFU/g of native microbiota; error bars indicate standard deviation from sample mean. Within microbial grouping, columns not sharing the same letter are statistically different ($p<0.05$). Limit of detection is 1 log_{10} CFU/g.

8.2.2.3 Native Microbiota on Parsley

For farm 1 (n=6, Temp=25 °C, ERH=74%), the counts of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative and heterofermentative LAB were 5.0±0.4, 4.6±0.4, 0.7±0.0, 5.0±0.7, 3.2±2.2, 4.6±0.1, 2.4±0.7, 3.4±1.4, 0.9±0.5, 3.6±0.3, 4.1±0.6 $\log_{10}$ CFU/g, respectively (Figure 8-4). For farm 2 (n=6, Temp=20 °C, ERH=48%), the counts of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative and heterofermentative LAB were 4.7±0.6, 4.8±0.6, 0.7±0.0, 4.5±0.7, 1.9±1.4, 4.2±0.5, 1.5±0.9, 3.3±0.5, 1.2±0.8, 2.3±0.3, 3.4±0.2 $\log_{10}$ CFU/g, respectively (Figure 8-4). Parsley from farm 1 bore greater numbers ($p<0.05$) of homofermentative and heterofermentative LAB versus farm 2. No differences between counts of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, and *E. coli* were observed in parsley ($p\geq0.05$).

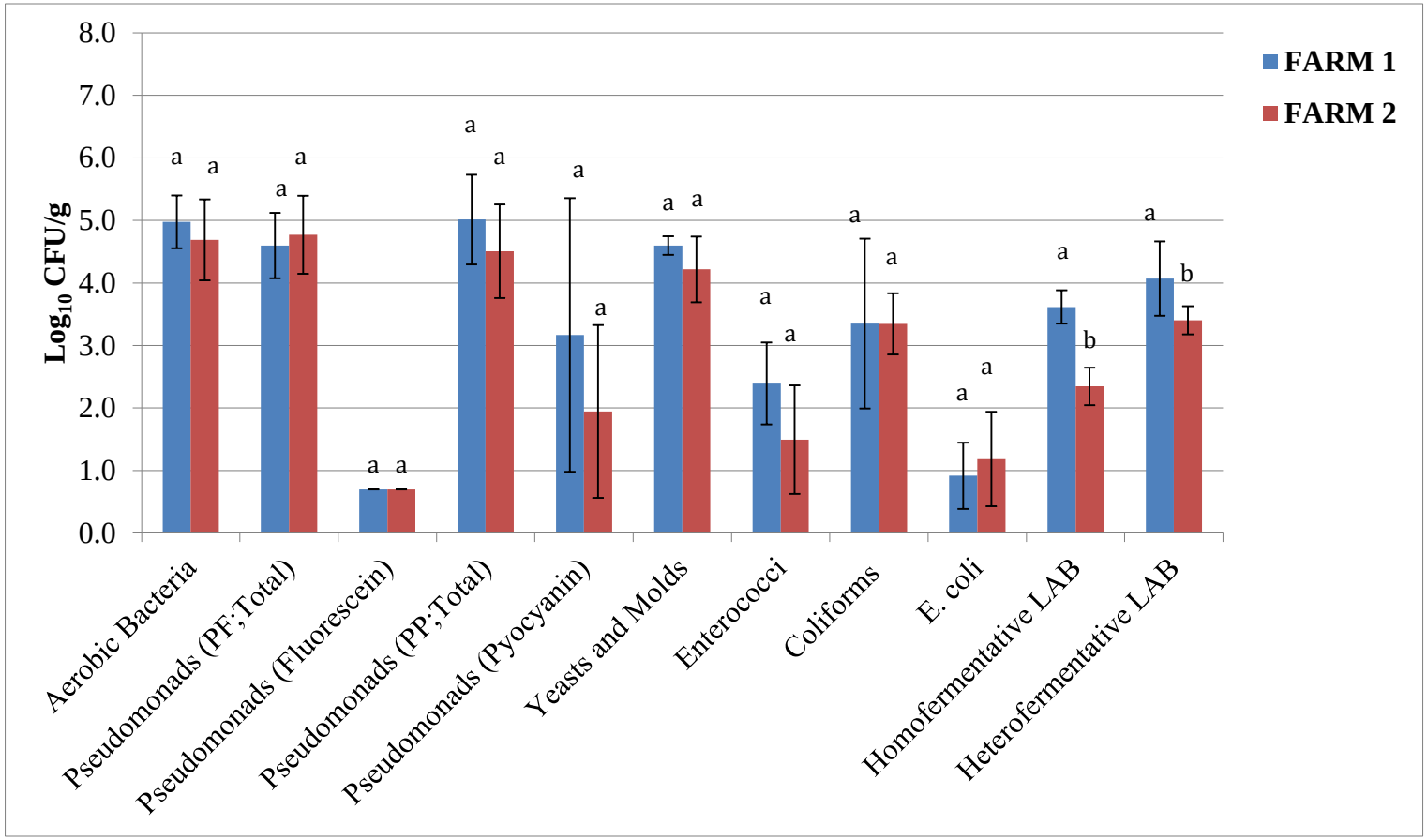

FIGURE 8-4. Log$_{10}$ CFU/g of native microbiota on surfaces of parsley samples from two Rio Grande valley farms. Columns represent mean log$_{10}$ CFU/g of native microbiota; error bars indicate standard deviation from sample mean. Within microbial grouping, means not connected by the same letter are statistically different ($p<0.05$). Limit of detection is 1 log$_{10}$ CFU/g.

8.2.2.4 Native Microbiota on Jalapeño Peppers

For spring harvest season (n=12), average microbial counts of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative and heterofermentative LAB across farm 1 (n=6, Temp=29 °C, ERH=62%) and farm 2 (n=6, Temp=36 °C, ERH=60%) were 3.9±0.5, 3.9±0.7, 0.5±0.9, 3.7±0.6, 0.6±1.0, 2.8±0.3, 0.2±0.0, 2.2±1.0, 0.2±0.1, 1.0±0.9, 1.7±1.1 $\log_{10}$ CFU/cm^2, respectively (Figure 8-5). For fall harvest season (n=12), the counts of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB obtained across farm 1 (Temp=22 °C, ERH=43%) and 2 (Temp=22 °C, ERH=78%) were 3.5±0.7, 3.5±0.8, 0.7±1.3, 3.5±0.8, 0.2±0.0, 2.6±1.0, 0.2±0.0, 1.9±1.1, 0.2±0.0, 1.8±0.9, 2.2±1.3 $\log_{10}$ CFU/ cm^2, respectively (Figure 8-5). Jalapeño pepper from spring harvest season bore greater numbers ($p<0.05$) of homofermentative LAB versus fall harvest season. The counts of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, and heterofermentative LAB were not different across two harvest seasons ($p\geq0.05$).

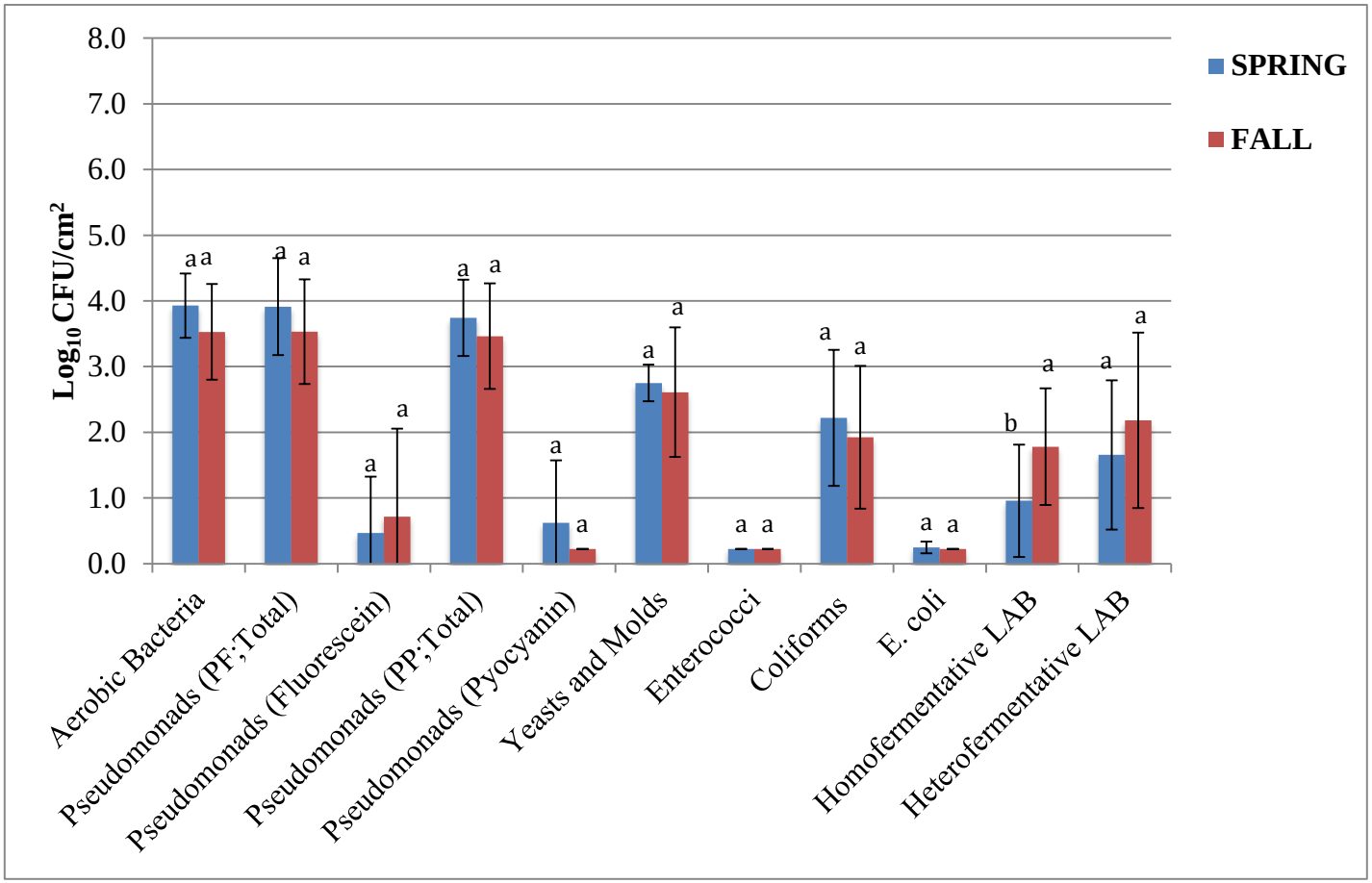

FIGURE 8-5. Log_{10} CFU/g of native microbiota on surfaces of jalapeño pepper samples from Rio Grande valley farms over two harvest seasons (n=12/season). Columns represent mean log_{10} CFU/cm^2 of native microbiota; error bars indicate standard deviation from sample mean. Within microbial grouping, means not connected by the same letter are statistically different ($p<0.05$). The limit of detection is 0.5 log_{10} CFU/cm^2

Fresh produce surfaces harbor a large variety of microbes including bacteria, yeast, molds and viruses. Bacteria found on surfaces of produce include both Gram-negative (e.g., *Pseudomonas*, *Escherichia coli*, *Klebsiella* spp., etc.) and Gram-positive organisms (e.g., *Micrococcus*, *Bacillus*, *Streptococcus*, LAB) *(140)*. Additionally, diverse molds (e.g., *Rhizopus*, *Aspergillus*, *Penicillium*, etc.) and yeasts (e.g. *Saccharomyces*, *Candida*, *Zygosaccharomyces*, etc.) are reported to inhabit produce surfaces *(101, 140)*. In this study, to obtain a native microorganism profile for field-harvested, non-washed Texas produce commodities, numbers of epiphytic microbiota on surfaces of leafy green produce were quantified as a function of farms in spring harvest season.

Overall, higher counts of microbial groupings were observed with leafy green samples collected at higher ambient temperature. For lettuce, counts of samples harvested at 24 °C and 81% RH were 0.4 to 1.4 $\log_{10}$ CFU/g higher than samples harvested at 16 °C and 51% RH. The same trend was also observed with parsley and spinach. In parsley, samples collected at 25 °C and 74% RH bore 0.7 to 1.4 $\log_{10}$ CFU/g greater than samples collected at 20 °C and 48% RH. For spinach, the counts of samples collected at 22 °C and 81 %RH were 0.5 to 3.6 $\log_{10}$ CFU/g higher than samples collected at 18 °C and 90 %RH, suggesting temperature may have been more important than RH in impacting numbers of microbiota. Although temperature seemed to play an important role in affecting numbers of epiphytes in leafy greens in this study, other factors also come into play. Factors affecting the populations of epiphytes can include pH (most microorganisms grow optimally at pH near neutrality), RH (Accumulation of

condensed moisture on plant surfaces due to high humidity can increase the level of microbiota), temperature (Mesophiles grow optimally at 20 to 40°C and higher temperature during harvest may favor growth of mesophilic organisms.), irrigation method (Complete coverage of the soil surface is obtained by flood irrigation and may have resulted in a higher microbial load.), available nutrients, antimicrobial substances, handling and processing practices (Cross-contamination by people or equipment can contribute to an elevated microbial load.) *(30)*, exposure to UV solar radiation (Microbial populations are normally larger on the abaxial leaf surfaces than the adaxial surfaces due to less exposure to UV radiation.) *(81)*, and surface characteristics of produce commodity (Commodities with rough and hydrophilic surface favors attachment of microorganisms.) *(244)*. Johnston et al. *(136)* reported the microbial populations of aerobic microbes (APC), enterococci, total coliforms, and *E. coli* from on surfaces of spinach from 13 farms in the southern United States to be 5.8±1.0, 2.1±0.9, 1.5±0.8, and 0.7±0.0 $\log_{10}$ CFU/g. From the same study, aerobic plate counts (APC), enterococci, total coliforms, and *E. coli* on the surfaces of parsley were 5.6±1.0, 2.5±0.9, 2.3±1.1, and 1.0±0.2 $\log_{10}$ CFU/g *(136)*. Maxy et al. *(158)* reported 4.8 $\log_{10}$ CFU/g of mesophilic aerobic bacteria on lettuce leaves. Ercolani et al. *(80)* reported numbers of aerobic bacteria, coliforms, and fecal streptococci to be 7.8, 4.8, and 3.4 $\log_{10}$ CFU/100g. Overall, the data from this current study are in agreement with previous studies.

8.2.3 Native Microbiota on Tomatoes

8.2.3.1 Native Microbiota on Tomato Skins

For spring harvest season (n=12), populations of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB across farm 1 (Temp=30°C, ERH=49%) and farm 2 (Temp=37°C, ERH=45%) were 3.4±0.9, 3.2±1.0, 0.7±0.7, 3.2±1.0, 1.0±1.1, 2.6±0.5, 0.3±0.3, 1.0±1.0, 0.2±0.1, 1.1±0.5, 1.5±1.0 $\log_{10}$ CFU/cm^2 respectively (Figure 8-6). For fall harvest season (n=12), the counts of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB obtained across farm 1 (Temp=21°C, ERH=86%) and farm 2 (Temp=22°C, ERH=77%) were 3.6±0.9, 3.8±0.9, 0.2±0.1, 3.7±0.9, 0.2±0.0, 2.8±1.0, 0.2±0.0, 0.9±0.8, 0.2±0.1, 1.9±1.5, 1.9±1.6 $\log_{10}$ CFU/cm^2 respectively (Figure 8-6). Jalapeño peppers from spring harvest season bore greater counts ($p<0.05$) of fluorescein-producing pseudomonads and pyocyanin-producing pseudomonads versus fall harvest season-recovered tomatoes. The numbers of aerobic bacteria, total pseudomonads from PF agar, total pseudomonads from PP agar, yeasts and molds, enterococci, total coliforms, *E. coli*, and heterofermentative LAB homofermentative LAB were not different between two harvest seasons ($p\geq0.05$).

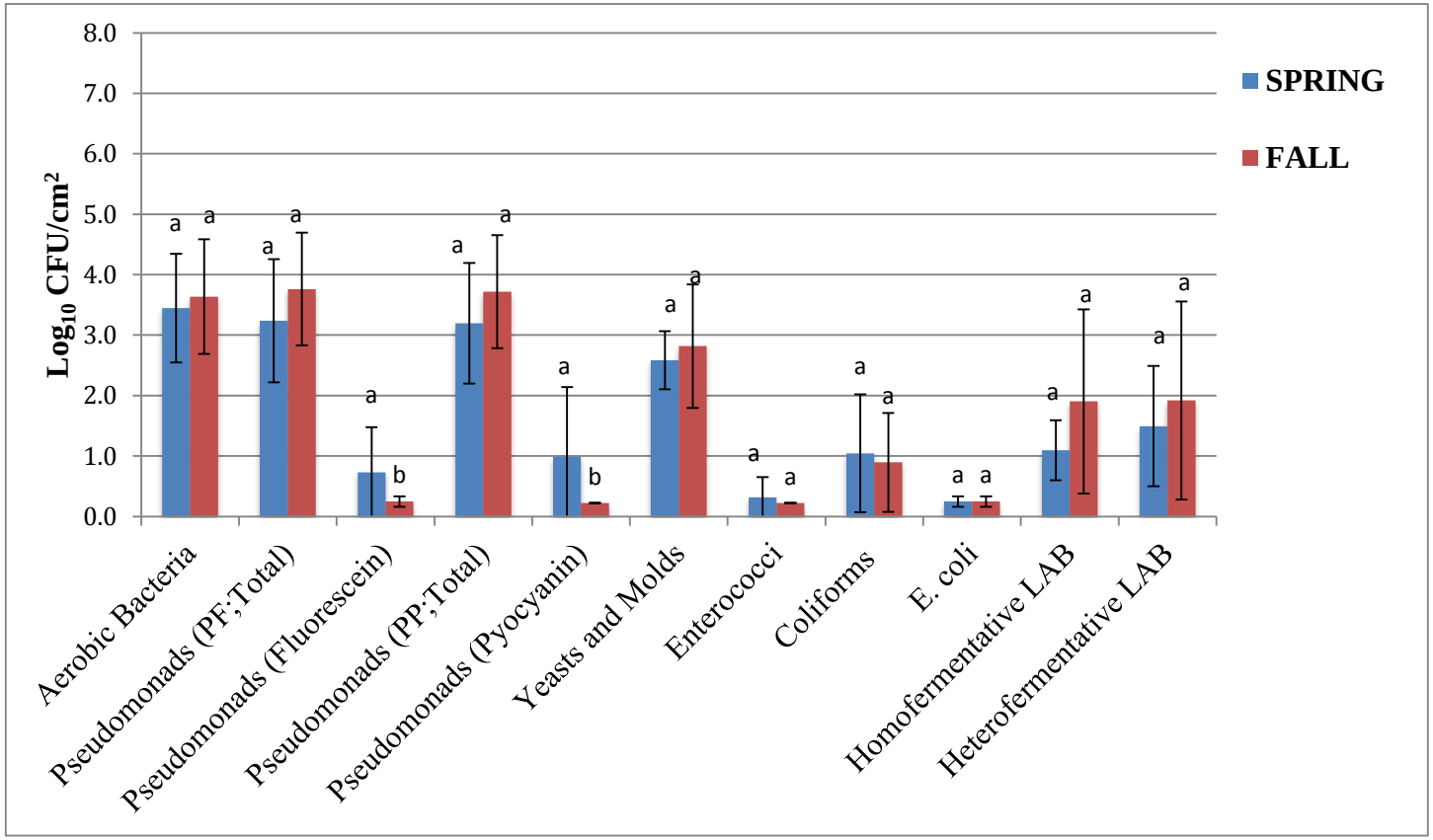

FIGURE 8-6. Log$_{10}$ CFU/g of native microbiota on skins of tomato samples from Rio Grande valley farms over two harvest seasons (n=12/season). Columns represent mean log$_{10}$ CFU/cm^2 of native microbiota; error bars indicate standard deviation from sample mean. Within microbial grouping, means not connected by the same letter are statistically different ($p<0.05$). Limit of detection is 0.5 log$_{10}$ CFU/cm^2.

8.2.3.2 Native Microbiota on Tomato Stem Scars

For spring harvest season (n=12), populations of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB from tomato stem scars across farm 1 (Temp=30 °C, ERH=49%) and farm 2 (Temp=37 °C, ERH=45%) were 5.4±0.4, 5.4±0.4, 1.9 ±1.3, 5.4±0.4, 2.9±1.8, 3.2±1.0, 0.9±0.0, 4.1±0.7, 1.1±0.5, 2.2±1.0, 3.9±0.8 $\log_{10}$ CFU/cm^2, respectively (Figure 8-7). For fall harvest season (n=12), the counts of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB obtained across farm 1 (Temp=21°C, ERH=86%) and farm 2 (Temp=22°C, ERH=77%) were 4.5±1.1, 4.5±1.1, 0.9±0.0, 4.4±1.1, 0.9±0.0, 2.8±1.1, 0.9±0.0, 3.0±1.7, 1.5±1.3, 2.7±1.7, 3.3±1.6 $\log_{10}$ CFU/cm^2, respectively (Figure 8-7). Higher counts of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads were observed from spring-obtained samples compared to samples from fall season ($p<0.05$). However, differences between counts of yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB were not observed between two harvest seasons ($p\geq0.05$).

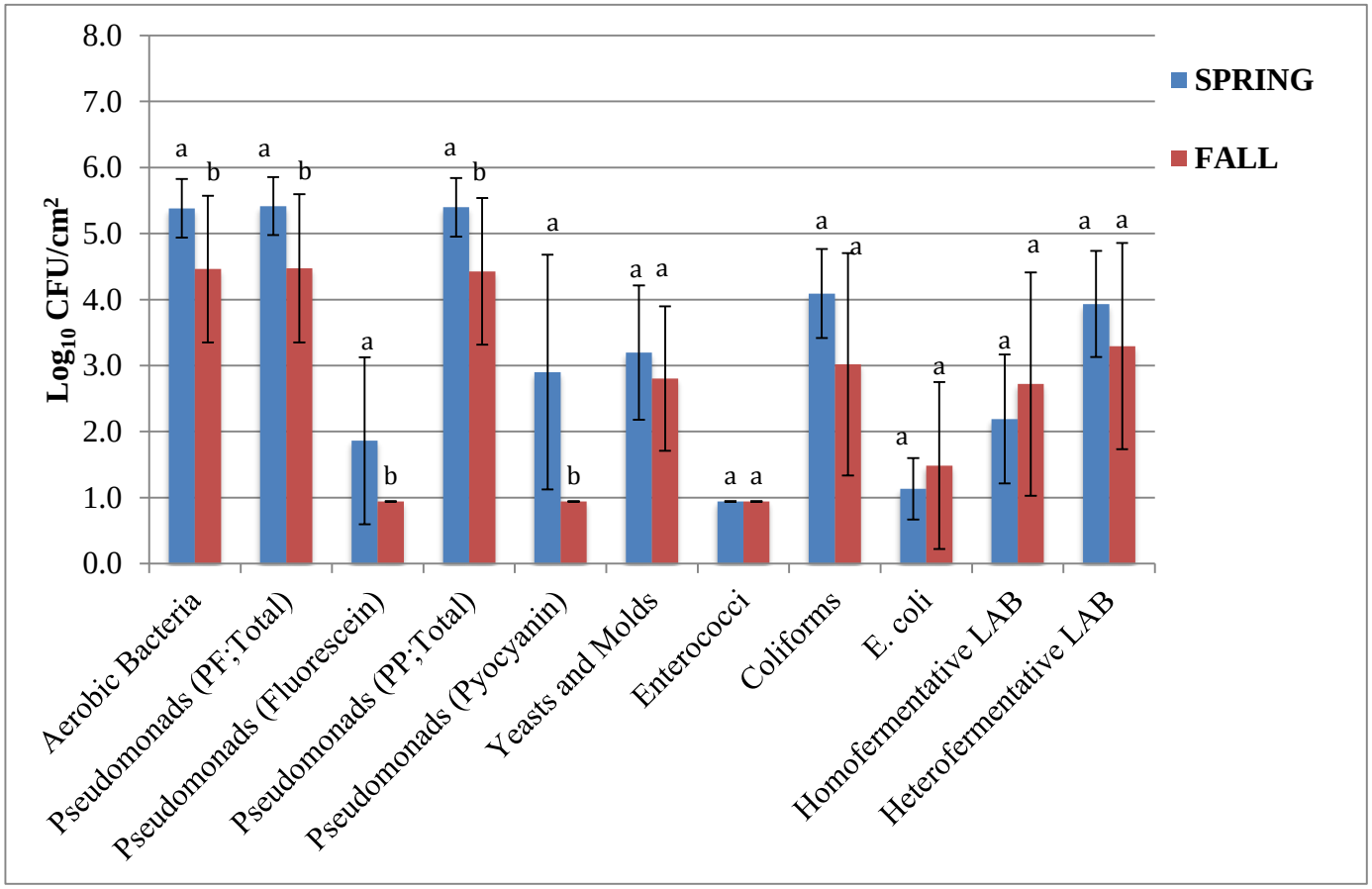

FIGURE 8-7. Log_{10} CFU/cm^2 of native microbiota on stem scars of tomato samples from Rio Grande valley farms over two harvest seasons (n=12/season). Columns represent mean log_{10} CFU/cm^2 of native microbiota; error bars indicate standard deviation from sample mean. Within microbial grouping, means not connected by the same letter are statistically different ($p<0.05$). Limit of detection: 0.9 log_{10} CFU/cm^2.

8.2.3.3 Native Microbiota on Tomato Skins versus Stem Scars

Across two harvest seasons (n=24 per two season), tomato stem scars bore greater numbers of aerobic bacteria, total pseudomonads from PF agar, total pseudomonads from PP agar, total coliforms, homofermentative LAB, and heterofermentative LAB ($p<0.05$) (Figure 8-8). Nevertheless, the differences between counts of fluorescein-producing pseudomonads, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, and *E. coli* between two tissue types were not observed ($p\geq0.05$) (Figure 8-8).

Log_{10} count differences of tomato stem scars versus skins over fall and spring harvest seasons (n=12 per season) are represented in Figure 8-9. The higher log_{10} count differences in spring harvest season were observed with aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads ($p<0.05$). However, the log_{10} count differences of yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB were not different between two seasons ($p\geq0.05$).

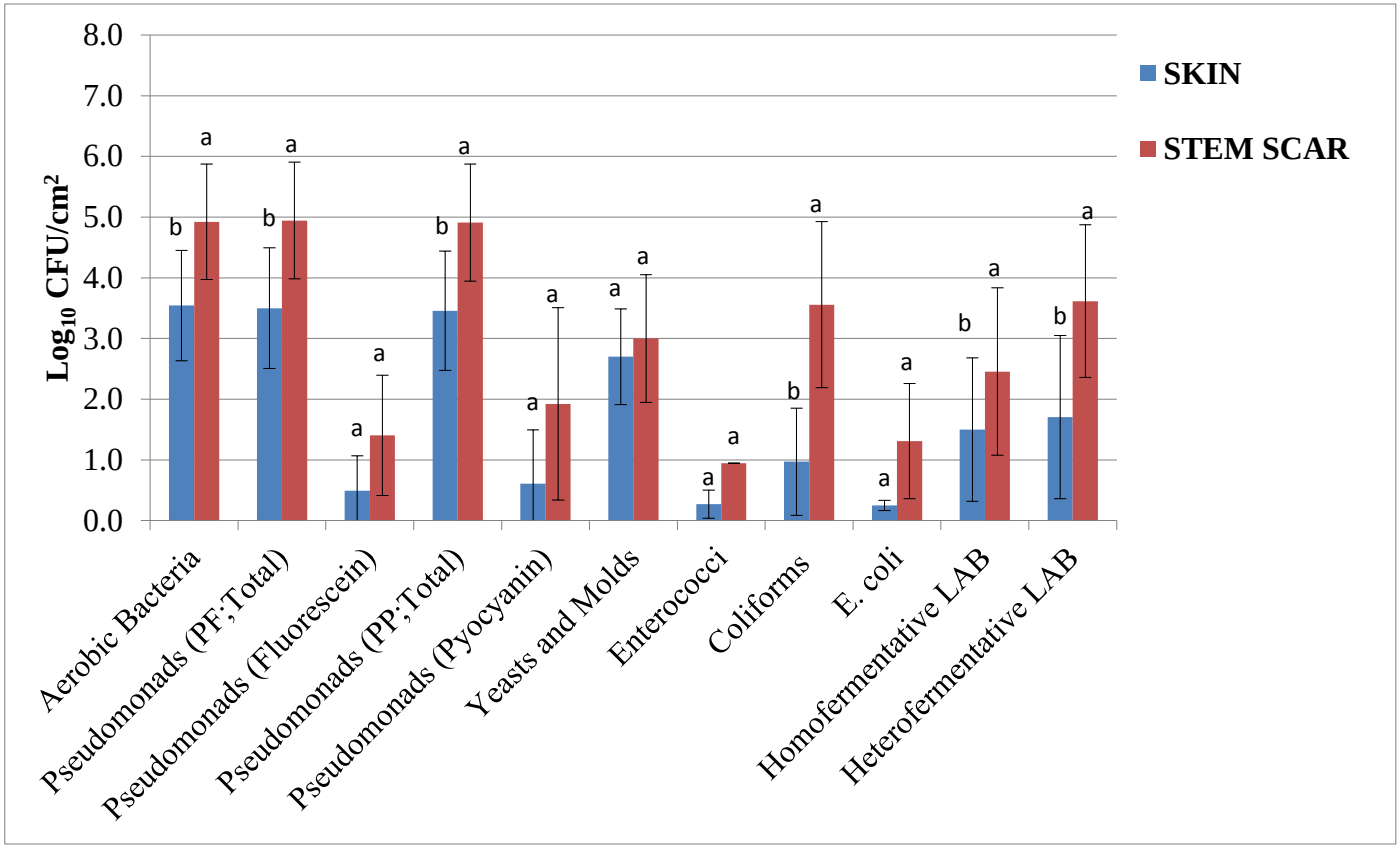

FIGURE 8-8. Log_{10} CFU/cm^2 of native microbiota on skins versus stem scars of tomato samples from Rio Grande valley farms across two harvest seasons (n=24). Columns represent mean log_{10} CFU/cm^2 of native microbiota; error bars indicate standard deviation from sample mean. Within microbial grouping, means not connected by the same letter are statistically different ($p<0.05$). The limit of detection for the microbial means from skins and stem scars are 0.5 and 0.9 log_{10} CFU/cm^2, respectively.

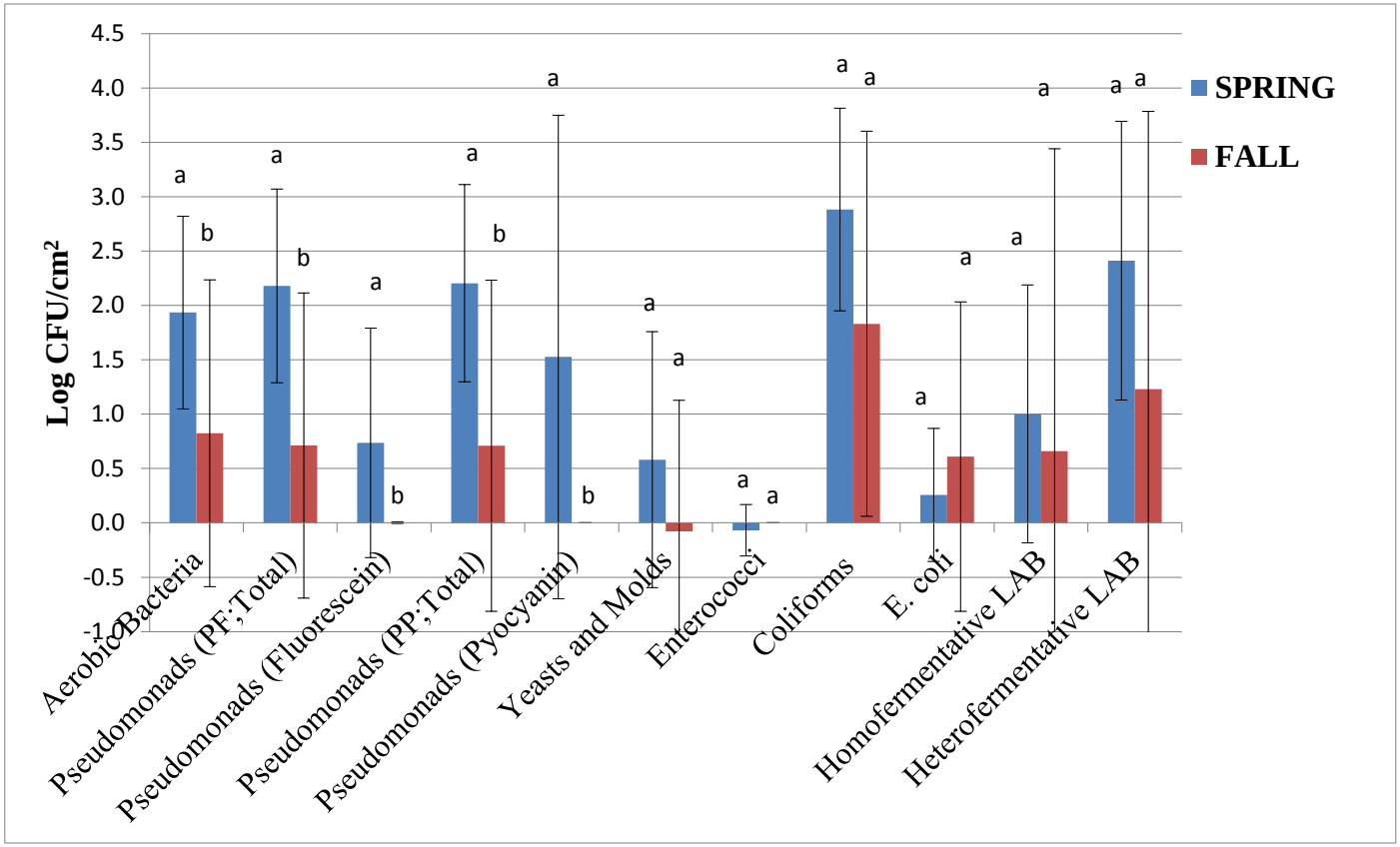

FIGURE 8-9. Log_{10} count differences of tomato stem scar and skin over two harvest seasons (n=12/season). Columns represent mean log_{10} count differences of native microbiota; error bars depict standard deviation from mean. Log_{10} count differences were calculated as log_{10} count on stem scar – log_{10} count on skin; differences were then averaged and standard deviations calculated. Within microbial grouping, means not connected by the same letter are statistically different ($p<0.05$).

8.2.4 Native Microbiota on Cantaloupes

8.2.4.1 Native Microbiota on Cantaloupe Rinds

For spring harvest season (n=12), the populations of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB across farm 1 (Temp=32°C, ERH=42%) and farm 2 (Temp=38°C, ERH=62%) were 5.9±0.5, 6.0±0.6, 1.1±1.3, 6.0±0.5, 1.4±2.1, 4.7±0.5, 1.8±1.0, 3.7±1.0, 1.6±1.1, 4.0±1.1, 4.5±0.7 $\log_{10}$ CFU/cm^2 respectively (Figure 8-10). For fall harvest season (n=12), the populations of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB obtained across farm 1 (Temp=26°C, ERH=65%) and farm 2 (Temp=26°C, ERH=79%) were 6.0±0.8, 6.0±0.8, 1.1±1.4, 6.0±0.8, 1.5±2.4, 4.4±0.5, 2.6±1.5, 4.5±0.9, 2.9±1.6, 4.7±0.9, 5.2±0.8 $\log_{10}$ CFU/cm^2 respectively (Figure 8-10). The counts of *E. coli* and heterofermentative LAB from fall harvest season were higher than those from spring harvest season ($p<0.05$). The differences between counts of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, homofermentative LAB, were not observed different between two harvest seasons ($p\geq0.05$).

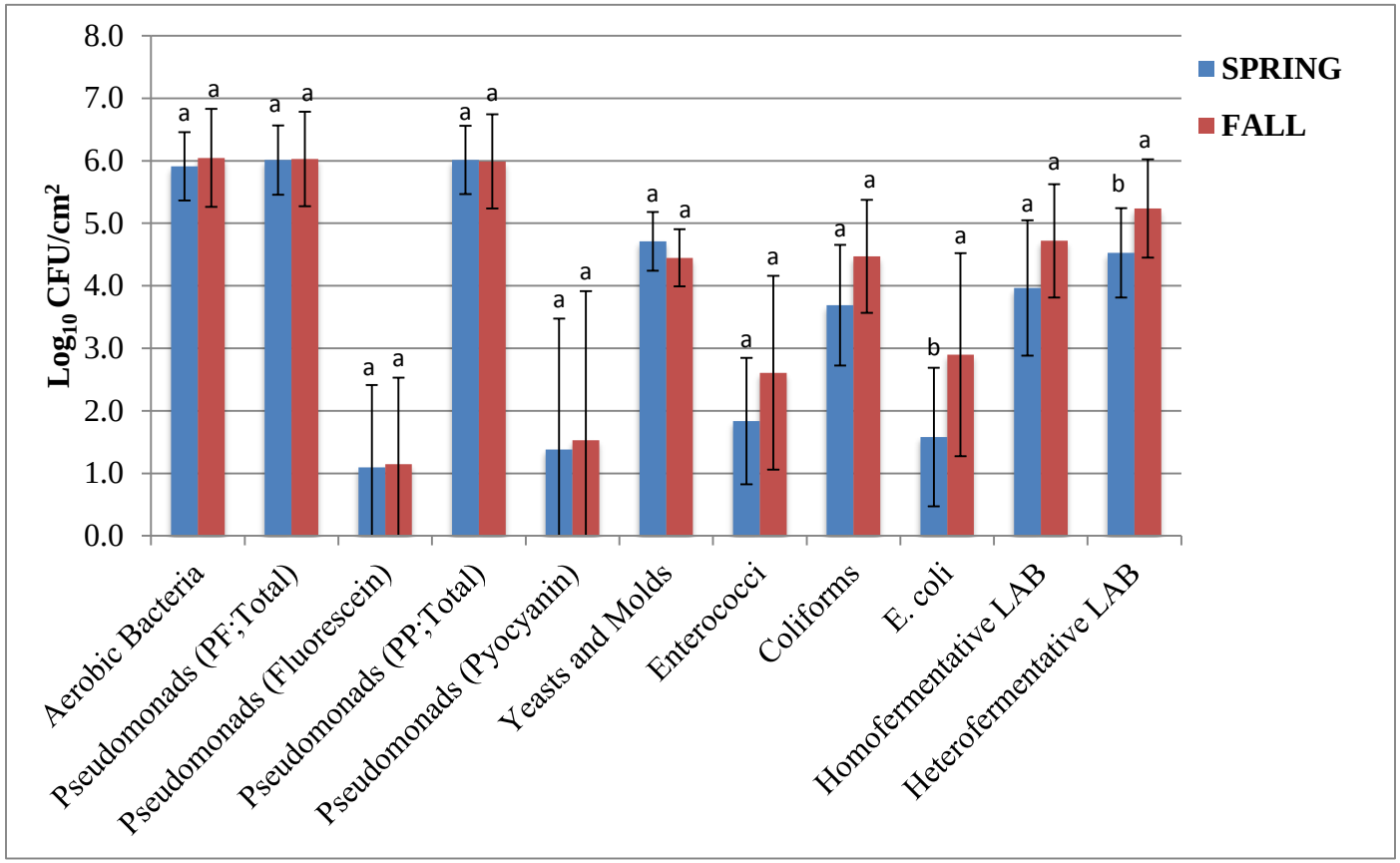

FIGURE 8-10. Log_{10} CFU/g of native microbiota on rinds of cantaloupe samples from Rio Grande valley farms over two harvest seasons (n=12/season). Columns represent mean log_{10} CFU/cm^2 of native microbiota; error bars indicate standard deviation from sample mean. Within microbial grouping, means not connected by the same letter are statistically different ($p<0.05$). The limit of detection is 0.5 log_{10} CFU/cm^2.

8.2.4.2 Native Microbiota on Cantaloupe Stem Scars

For spring harvest season (n=12), average counts of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB across farm 1 (Temp=32°C, ERH=42%) and farm 2 (Temp=38°C, ERH=62%) were 7.0±0.6, 6.9±0.6, 1.5±2.1, 7.0±0.7, 1.5±1.9, 5.4±0.7, 2.1±1.6, 4.8±0.9, 2.0±1.2, 5.5±1.1, 5.4±1.2 $\log_{10}$ CFU/cm^2 respectively (Figure 8-11). For fall harvest season (n=12), the average populations of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB obtained across farm 1 (Temp=26°C, ERH=65%) and farm 2 (Temp=26°C, ERH=79%) were 6.6±0.8, 6.6±0.7, 1.8±2.0, 6.4±0.8, 3.0±2.5, 3.9±1.4, 2.3±2.0, 5.1±1.0, 3.4±1.7, 4.9±1.7, 5.7±1.0 $\log_{10}$ CFU/cm^2 respectively (Figure 8-11). Cantaloupe stem scars from spring season bore greater counts of yeasts molds versus fall season. ($p<0.05$). However, *E. coli* populations from fall season were higher than from spring season-harvested samples ($p<0.05$). Numbers of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, enterococci, total coliforms, homofermentative LAB, and heterofermentative LAB did not differ between two harvest seasons ($p\geq0.05$).

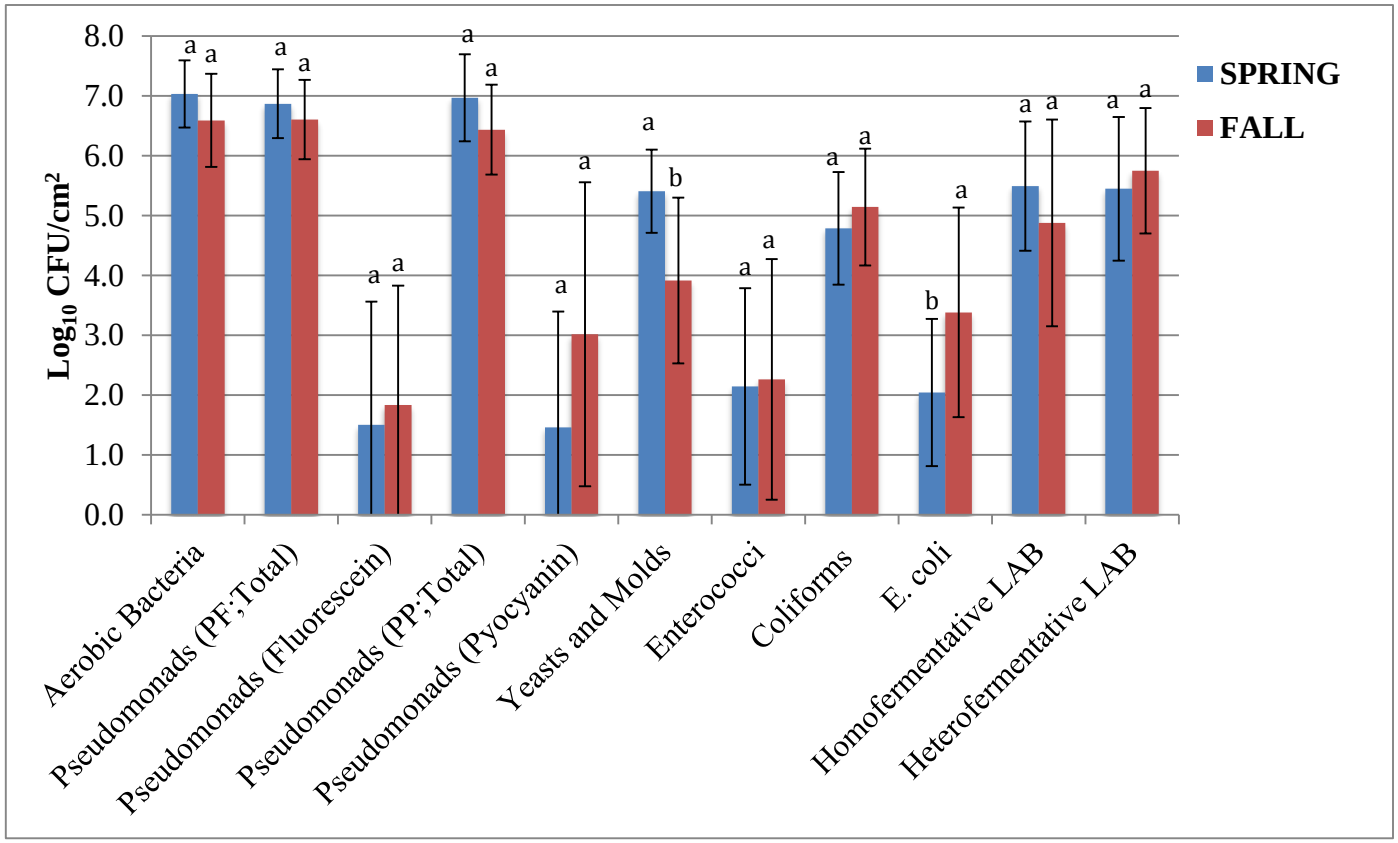

FIGURE 8-11. Log_{10} CFU/g of native microbiota on stem scars of cantaloupe samples from Rio Grande valley farms over two harvest seasons (n=12/season). Columns represent mean log_{10} CFU/cm^2 of native microbiota; error bars indicate standard deviation from sample mean. Within microbial grouping, means not connected by the same letter are statistically different ($p<0.05$). The limit of detection is 0.9 log_{10} CFU/cm^2

8.2.4.3 Native Microbiota on Cantaloupe Rinds versus Stem Scars

Across two harvest seasons (n=24 per two seasons), numbers of aerobic bacteria, total pseudomonads from PF agar, total pseudomonads from PP agar, coliforms, homofermentative LAB, and heterofermentative LAB from cantaloupe stem scars were higher than from cantaloupe rinds ($p<0.05$) (Figure 8-12). Nevertheless, counts of fluorescein-producing pseudomonads, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, and *E. coli* did not differ between two tissue types ($p\geq0.05$) (Figure 8-12).

Figure 8-13 depicts $\log_{10}$ count differences of cantaloupe stems scars versus skins over two harvest seasons (n=12 per season). Higher $\log_{10}$ count difference of cantaloupe stems scars versus skins in spring was observed with yeasts and molds ($p<0.05$). However, $\log_{10}$ count differences of other microbial groupings did not differ between fall and spring harvest season ($p\geq0.05$).

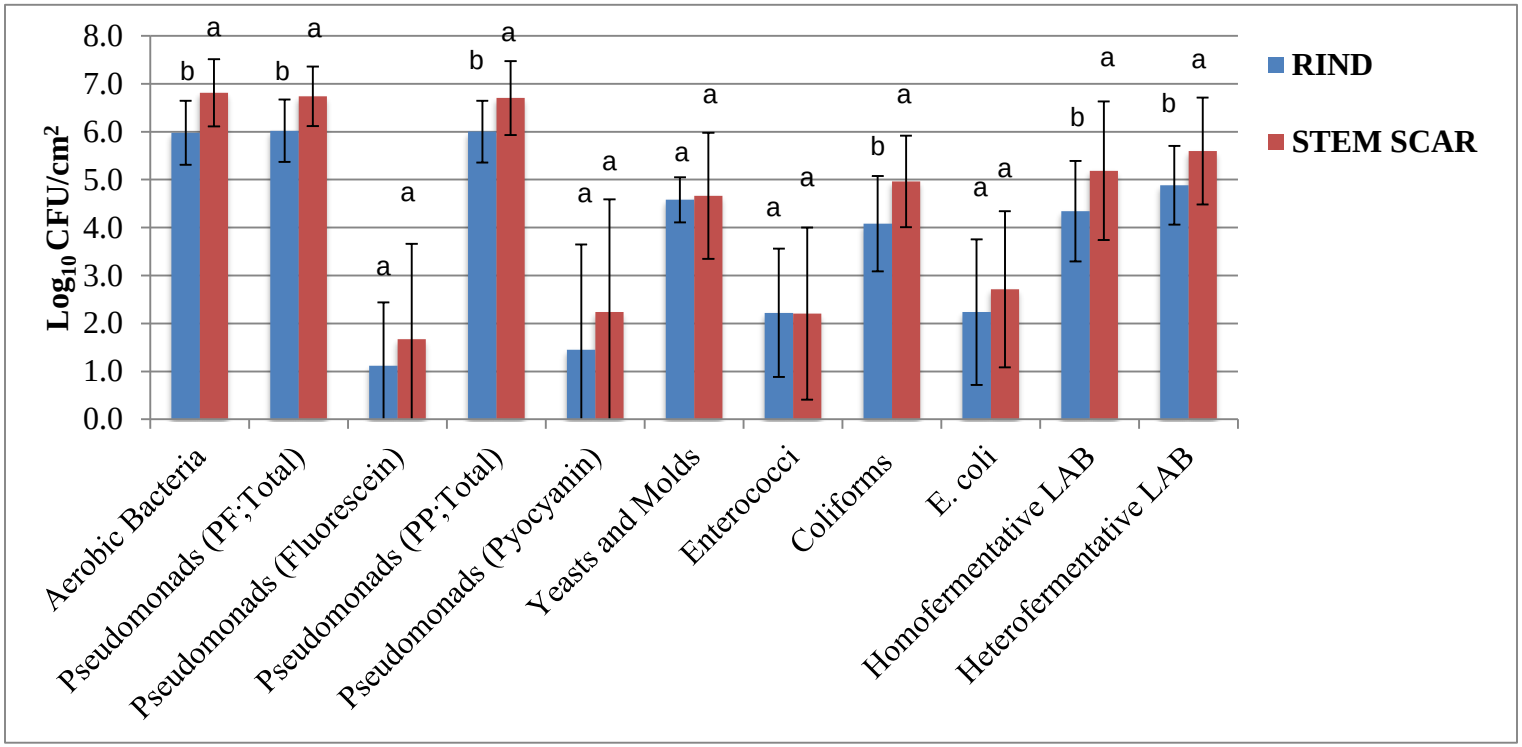

FIGURE 8-12. Log_{10} CFU/cm^2 of native microbiota on skins versus stem scars of cantaloupe samples from Rio Grande valley farms over two harvest seasons (n=24). Columns represent mean log_{10} CFU/cm^2 of native microbiota; error bars indicate standard deviation from sample mean. Within microbial grouping, means not connected by the same letter are statistically different ($p \geq 0.05$). The limits of detection for the microbial means from skins and stem scars are 0.5 and 0.6 log_{10} CFU/cm^2 respectively.

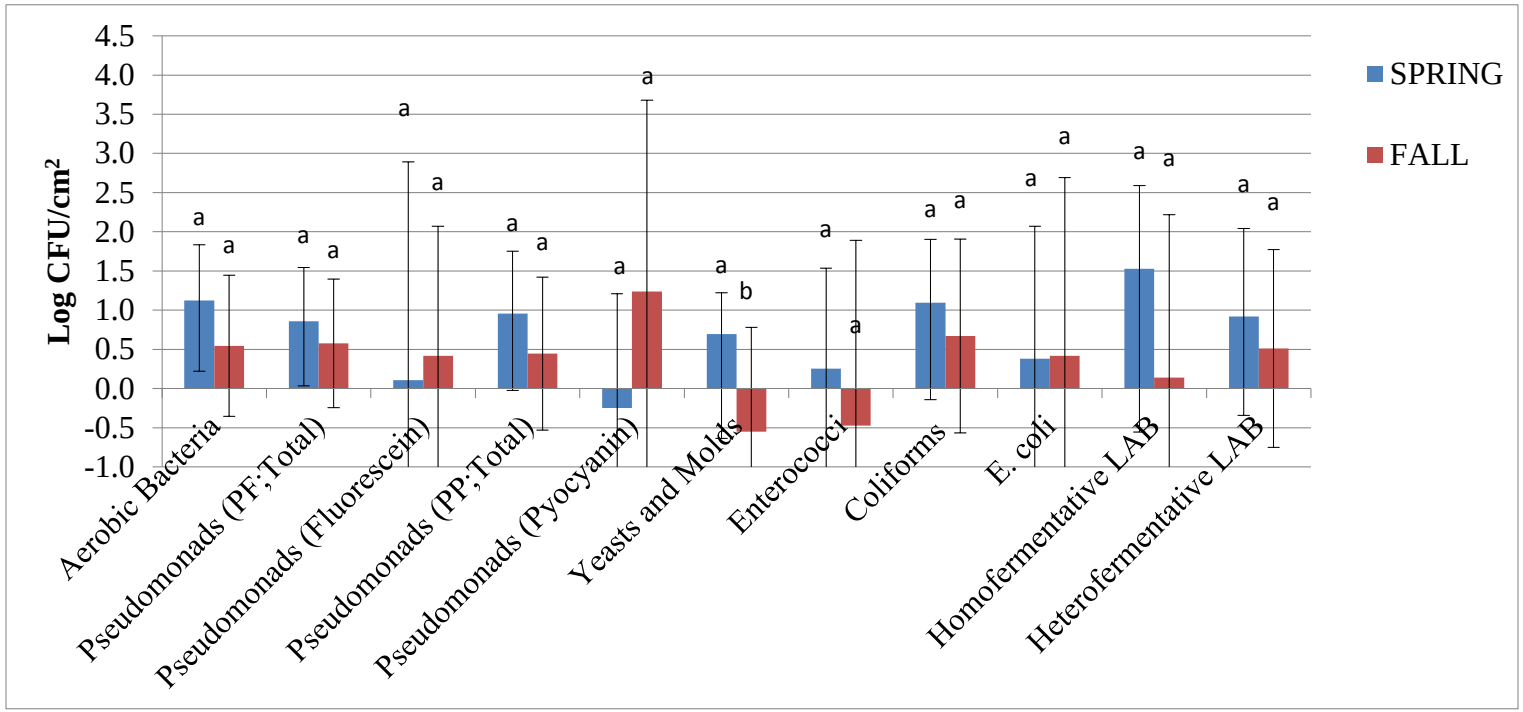

FIGURE 8-13. Log_{10} count differences of cantaloupe stem scars and rinds over two harvest seasons (n=12/season). Columns represent mean log_{10} count differences of native microbiota; error bars depict standard deviation from mean. Log_{10} count differences were calculated as log_{10} count on stem scar – log_{10} count on skin; differences were then averaged and standard deviations calculated. Within microbial grouping, means not connected by the same letter are statistically different ($p<0.05$).

8.2.4.4 Native Microbiota on Tomatoes versus Cantaloupes

Across two harvest seasons (n=24 per two seasons), numbers of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB from cantaloupe stem rinds were higher than from tomato rinds ($p<0.05$) (Table 8-1). However, populations of pyocyanin-producing pseudomonads were not different between two produce commodities ($p\geq0.05$) (Table 8-1).

For stem scars, across two harvest seasons (n=24), the average counts of aerobic bacteria, total pseudomonads from PF agar, total pseudomonads from PP agar, yeasts and molds, enterococci, coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB from cantaloupes were higher than from tomatoes ($p<0.05$). Nevertheless, populations of fluorescein-producing and pyocyanin-producing pseudomonads did not differ between tomatoes and cantaloupes ($p\geq0.05$) (Table 8-1).

TABLE 8-1. Microbial loads (log_{10} CFU/cm^2) on produce skin/rind and stem scar from Texas-harvested tomatoes and cantaloupes over two harvest seasons (n=24).

Microbial Grouping	Surface-Specific Mean Microbial Loads			
	Tomato		Cantaloupe	
	Skin	Stem Scar	Rind	Stem Scar
Aerobic Bacteria	$3.5 \pm 0.9^{a,x}$	$4.9 \pm 1.0^{b,\alpha}$	$6.0 \pm 0.7^{a,y}$	$6.8 \pm 0.7^{b,\beta}$
Pseudomonads (*Pseudomonas* Agar F)	$3.5 \pm 1.0^{a,x}$	$4.9 \pm 1.0^{b,\alpha}$	$6.0 \pm 0.6^{a,y}$	$6.7 \pm 0.6^{b,\beta}$
Fluorescein-Producing Pseudomonads	$0.5 \pm 0.6^{a,x}$	$1.4 \pm 1.0^{a,\alpha}$	$1.1 \pm 1.3^{a,y}$	$1.7 \pm 2.0^{a,\alpha}$
Pseudomonads (*Pseudomonas* Agar P)	$3.5 \pm 1.0^{a,x}$	$4.9 \pm 1.0^{b,\alpha}$	$6.0 \pm 0.6^{a,y}$	$6.7 \pm 0.8^{b,\beta}$
Pyocyanin-Producing Pseudomonads	$0.6 \pm 0.9^{a,x}$	$1.9 \pm 1.6^{a,\alpha}$	$1.5 \pm 2.2^{a,x}$	$2.2 \pm 2.3^{a,\alpha}$
Yeasts and Molds	$2.7 \pm 0.8^{a,x}$	$3.0 \pm 1.1^{a,\alpha}$	$4.6 \pm 0.5^{a,y}$	$4.7 \pm 1.3^{a,\beta}$
Enterococci	$0.3 \pm 0.2^{a,x}$	$0.9 \pm 0.0^{a,\alpha}$	$2.2 \pm 1.3^{a,y}$	$2.2 \pm 1.8^{a,\beta}$
Coliforms	$1.0 \pm 0.9^{a,x}$	$3.6 \pm 1.4^{b,\alpha}$	$4.1 \pm 1.0^{a,y}$	$5.0 \pm 1.0^{b,\beta}$
Escherichia coli	$0.2 \pm 0.1^{a,x}$	$1.3 \pm 0.9^{a,\alpha}$	$2.2 \pm 1.5^{a,y}$	$2.7 \pm 1.6^{a,\beta}$
Homofermentative LAB	$1.5 \pm 1.2^{a,x}$	$2.5 \pm 1.4^{b,\alpha}$	$4.3 \pm 1.1^{a,y}$	$5.2 \pm 1.4^{b,\beta}$
Heterofermentative LAB	$1.7 \pm 1.3^{a,x}$	$3.6 \pm 1.3^{b,\alpha}$	$4.9 \pm 0.8^{a,y}$	$5.6 \pm 1.1^{b,\beta}$

*Significant differences amongst mean log_{10} counts were determined by ANOVA and means were separated using Student's t-test ($p<0.05$).

^{a,b}Mean log_{10} counts within a row, within the same commodity (tomato or cantaloupe), with differing superscripts are statistically different ($p<0.05$).

^{x,y}Mean log_{10} counts within a row, across commodity, for skin and rind, with differing superscripts are statistically different ($p<0.05$).

$^{\alpha,\beta}$Mean log_{10} counts within a row, across commodity, for stem scar, with differing superscripts are statistically different ($p<0.05$).

Numbers of epiphytic microbiota on surfaces of jalapeno peppers, tomatoes, and cantaloupes were determined as a function of harvest seasons. For tomatoes and cantaloupes, the numbers of epiphytes as a function of different tissue types, and commodities were also studied. The ambient temperature in spring and fall ranged from 29°C to 38°C and 21°C to 26°C respectively. The relative humidity ranged from 42%RH to 62%RH in spring and 43%RH to 86%RH in fall. In tomatoes, higher counts of certain microbial groupings were observed in spring harvest season that was warmer than fall season. For tomato skins, the counts of fluorescein-producing and pyocyanin-producing pseudomonads in spring were 0.5 and 0.8 $\log_{10}$ CFU/cm^2 higher than in fall respectively. For tomato stem scars, higher counts (0.9 to 2 $\log_{10}$ CFU/cm^2 higher) of aerobic bacteria, total pseudomonads from PF and PP agar, fluorescein-producing and pyocyanin-producing pseudomonads were also obtained in spring season. However, the same trend was not observed with cantaloupe and pepper samples. In cantaloupe rinds, higher counts of *E. coli* (1.3 $\log_{10}$ CFU/cm^2 higher) and heterofermentative LAB (0.7 $\log_{10}$ CFU/cm^2 higher) were obtained in fall versus spring. For cantaloupe stem scars, yeasts and molds from spring-obtained samples were 1.5 $\log_{10}$ CFU/cm^2 higher than fall-obtained samples. However, fall-obtained samples bore 1.4 $\log_{10}$ CFU/cm^2 of *E. coli* higher than spring-obtained samples. For pepper, the only count difference between two seasons was observed in homofermentative LAB with 0.8 $\log_{10}$ CFU/cm^2 higher in fall season.

In general, stem scars bore greater numbers of microbes versus skins/rinds for most microbial groupings over both commodities. Excepting yeasts and molds, the

counts of all microbial groupings were higher in tomato stem scars versus skins. For cantaloupe, higher counts of aerobic bacteria, pseudomonads from PF and PP agar, fluorescein-producing and pyocyanin-producing pseudomonads, coliforms, homofermentative and heterofermentative LAB were higher in stem scars. Higher counts on stem scars are likely due to their porous nature, which can provide for greater microbial attachment area *(117)*. Also, due to roughness of stem scars, microorganisms could be shielded by entrapped air, debris, and plant surface structures *(180)*. Wang et al. *(244)* reported surface roughness of cantaloupe to be 14.18±0.25 μm, while Fernandes et al. *(100)* reported roughness of tomato to be 2.88±2.15 μm. Therefore, unlike smooth surfaces of tomato skins, netting and roughness on cantaloupe rinds may provide for increased microbial attachment *(41, 72)*.

Cardenas et al.*(40)* reported populations on skins of jalapeño pepper from retail markets in Monterrey, Mexico, to be 4.4 $\log_{10}$ CFU/g for total mesophiles, 3.3 $\log_{10}$ CFU/g for total coliforms, and 1.7 $\log_{10}$ CFU/g for fecal coliforms; the counts are slightly higher than those from my current study. Total mesophiles of 3.2 $\log_{10}$ CFU/g and 2.6 $\log_{10}$ CFU/g for total coliforms in peppers were also reported by Cardenas et al. *(40)*. Johnston et al. *(136)* reported numbers of aerobic plate count (APC), enterococci, total coliforms, and *E. coli* on surfaces of spinach from farms in the southern United States to be 5.8±1.0, 6.6±1.1, 4.1±1.2, 3.0±1.3, and 1.5±1.1 CFU/g, respectively. In general, data from my study seem to be in line with previous studies. The native microorganisms profile will be useful for prediction of the production system as well as developing pre-harvest biocontrol methods using pathogen antagonists. This research

suggests need for further analysis of the correlations between harvest conditions and microbiological profile on produce commodities.

CHAPTER IX

INTERNALIZATION IN FRESH PRODUCE

9.1 Materials and Methods

9.1.1 Dye Internalization in Tomatoes with Aid of Temperature and Pressure Difference

The experiment was conducted to test the hypothesis that the intact tomato would provide resistance to dye penetration through stem scars and more dye penetration would be observed with the non-intact tomato. Roma tomatoes were obtained from a local grocer. Prior to the experiment, tomato samples were equilibrated to room temperature (~25°C) for 24 hr. One set of tomatoes was horizontally cut in half while a second set of fruits was kept intact. Intact and non-intact tomato samples were completely submerged in 2.0 liters 5 °C Brilliant Blue FCF dye (Sigma Aldrich Co., St. Louis, MO) for 2 h. After submersion, tomato samples were cut in half through stem scars and dye penetration depths were measured using a caliper. The experiment was performed in triplicate; each replicate was done in duplicate (n=6).

9.1.2 Microbial Internalization in Tomatoes without Aid of Temperature and Pressure Difference

Rifampicin-resistant (Rif^R) *E. coli* K12 was obtained from the Food Microbiology Laboratory culture collection (Department of Animal Science, Texas A&M University, College Station, TX) and maintained on slants of tryptic soy agar (TSA; Becton, Dickinson and Co.) at 5 °C. Working culture of the bacterial pathogen surrogate was prepared by transferring a loopful of culture from TSA slants to 10 ml

TSB and incubating at 35 °C for 24 h. After incubation, the culture was transferred into sterile 10 ml TSB and incubated at 35 °C for 24 h. Stem-intact vine tomatoes were purchased from a local grocer and surface sanitized using sterile distilled water and 70% ethanol; samples were then air dried for 1 h. Tomato stems were inoculated with 100 µl 7.0±0.1 $\log_{10}$ CFU/ml Rif^R *E. coli* K12. After 1 h attachment, stems were aseptically removed using sterile scalpel and forceps to expose stem scars on tomatoes. Samples were then held at 25 °C for 24 h to allow for microbial internalization through stem scars. Afterward, stem scars were aseptically excised into 3 pieces with 0.6 cm height each (top, middle, and bottom) relative to the intact stem. Stem scar samples were placed in individual stomacher bags and hand-pummeled with 25 ml 0.1% peptone diluent for 1 min to determine potential microbial internalization depth. Resulting samples were serially diluted and spread on TSA containing 0.1g/liter rifampicin (Sigma Aldrich Co., St. Louis, MO) (TSAR). TSAR plates were incubated at 35 °C for 24 h and colonies enumerated. The experiment was performed in triplicate with duplicate samples for each replicate (n=6).

9.1.3 Scanning Electron Microscopy Observation of Tomato Stem Scars

Uninoculated stem scar samples were also visualized using scanning electron microscopy (SEM) to determine physical characteristics of stem scars. The top part (at the stem end), vertical cross section, lateral border, and bottom border of an intact stem scar sample was aseptically excised using a sterile scalpel and forceps. The sample preparation procedure was adapted from Pao et al. *(181)*. Stem scar samples were aseptically excised and were fixed in phosphate buffer containing 3% glutaraldehyde

(Ted Pella Inc., Redding, CA) pH 7.0 for 48 h to the maintain structural integrity of the samples. After fixation, samples were washed 3 times with 0.1 M potassium phosphate buffer (Sigma Aldrich Co.) and were dehydrated with gradual concentrations (10, 25, 50, 75, 90, and 100%) of ethanol (Koptec, King of Prussia, PA). After overnight vacuum drying, samples were placed on aluminum stubs with carbon sticky tape and then sputter coated with platinum using a Hummer I Sputter Coater (Anatech Ltd., Union City,CA) for 4 min at 10 mA. Samples were observed using a JEOL 6400 scanning electron microscope (JEOL USA, Peabody, MA).

9.1.4 Statistical Analyses

Prior to statistical analysis, *E. coli* count data were logarithmically transformed (base 10). Statistical analyses of the dye penetration data and logarithmically-transformed data were performed using JMP v10.0.0 (SAS Institute Inc., Cary, N.C.). Significant differences ($p<0.05$) amongst mean dye penetration and $\log_{10}$ counts of *E. coli* were determined by ANOVA and means were separated using Student's t-test.

9.2 Results and Discussion

9.2.1 Dye Internalization

Before the experiment, it was hypothesized that intact tomato would provide resistance to dye penetration through stem scars as a function of temperature and pressure differential. Cutting tomato was hypothesized to reduce internal pressure of tomato, resulting in less resistance to dye internalization. Thus, more dye penetration through stem scars would occur in cut tomato samples versus intact tomato samples. However, in this study, more dye penetration through stem scar was observed with intact

tomatoes versus cut tomatoes ($p<0.05$) (Figure 9-1). Dye penetrations in intact and non-intact tomatoes were 1.71±1.36 cm and 0.10±0.06 cm respectively (Table 9-1).

TABLE 9-1. Dye penetration (cm) through stem scars of intact and non-intact tomato samples

Tomato sample	Dye penetration
Intact	1.71±1.36[a]
Non-intact	0.10±0.06[b]

Dye penetrations not connected by the same letter are significantly different ($p<0.05$) samples.

FIGURE 9-1. Dye penetration (cm) through stem scars of intact (left) and non-intact (right) tomato samples.

In this study, dye internalization through tomato stem scar was studied as a surrogate of microbial internalization. It has been reported that submerging warm pieces of produce in cold microbe/dye solution resulted in internalization of the solution or microbe into produce *(17, 18, 78, 181, 255)*. This phenomenon is dictated by the ideal gas law since submerging a warm fruit in a cooler solution causes contraction of gas in the intercellular spaces resulting in a hydrostatic pressure differential that allows external water to be drawn inside the fruit *(17, 255)*. Therefore, plants and human pathogens can be internalized into intact fruits if inappropriate practices were used with flume water or dump tank systems *(182)*. Factors contributing to susceptibility of pathogen internalization into intact fruit can also include time delay after stem scar removal and water submersion (old stem scars are less porous than fresh stem scars) *(17, 216)*, porosity of stem scar, physical and chemical characteristics of vascular bundles *(255)*. For non-intact tomato, cutting tomato may have exposed the internal part of the fruit to the dye/environment resulting in reduced pressure differential between the fruit and a dye solution and thus prevented dye from infiltrating the fruit through stem scars efficiently. However, dye can penetrate through flesh at a greater extent and pathogen internalization into non-intact fruits can also occur if contaminated wash water is used. Once internalized, pathogens are protected by tomato tissues, making removal or inactivation difficult *(255)*. To reduce pathogen internalization risks, preventing pathogen internalization is of a paramount importance *(255)*. In typical tomato packing houses, the current postharvest handling practices includes immersion of tomatoes for up

to 2 min in a heated chlorinated dump tank wash water that is at least 5.6 °C higher than tomato pulp temperature *(255)*.

9.2.2 Microbial Internalization in Tomatoes without Aid of Temperature and Pressure Difference and SEM Observation

This research studied the extent of *E. coli* K12 internalization through tomato stem scars by post-harvest inoculation without aid of temperature or pressure difference to determine the depth to which internalized organisms may travel within channels in tomato stems. *E. coli* K12 (2.8±1.6 $\log_{10}$ CFU/cm^3) were recovered from the top stem scar samples (0.6 cm depth) but not the middle and the bottom pieces (Table 9-2). Without aid of temperature or pressure differential, it is hypothesized that bacteria may have infiltrated tomato stem scars as a function of gas and fluid exchange. Removal of stem may have released fruit exudate that eventually washed microbial cells surrounding the stem area resulting in microbial cell suspension. Once water from the microbial cell suspension evaporated, microbial cells could travel down the stem scar and adhere to the scar walls. SEM images of internal stem scar samples showed vascular bundle structures approximately 100 μm in length and 15 μm diameter (Figure 9-2). Vascular bundles are the natural channels that allow transportation of water and nutrients in plant through capillary action *(73, 181)*. Bacterial size vary depending on species, but in general, range from 0.5 to 1 μm in diameter or width. Therefore, bacteria can infiltrate plant stem scars and move along vascular bundles, which are larger in size. These results suggest deposit of aqueous microbial suspension onto tomato stem could result in opportunity for internalization through vascular bundles structures, however internalized microbes

are unable to travel to cells deep within the stem probably due to hydrophobic nature of stem scars *(17)* and also the presence of air in vascular bundles that prevented the microbe from travelling deeply.

TABLE 9-2. Populations (log_{10} CFU/cm^3) of internalized *Escherichia coli* K12 in tomato stem scar samples.

Stem scar sample	*E. coli* K12 population
Top	2.8±1.6[a]
Middle	ND[b]
Bottom	ND

[a] Populations not connected by the same letter are significantly different ($p<0.05$). Undetected populations were assigned a value (1.6 log_{10} CFU/cm^3) halfway between zero and the assay detection limit for statistical analysis.
[b] ND: not detected.

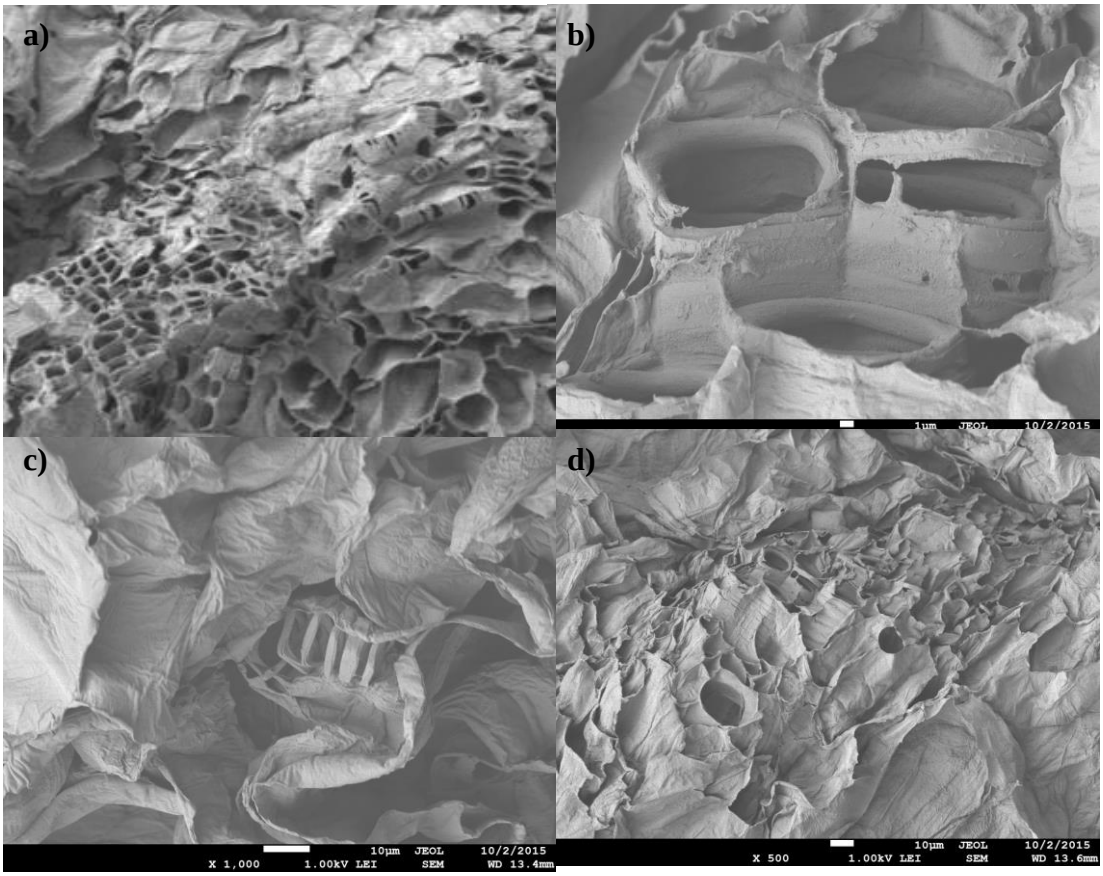

FIGURE 9-2. Scanning electron micrographs of vascular bundle structures. of a) top part (at the stem end) of tomato stem scar (100X, 200µm), b) lateral border of tomato stem scar (2000X, 10 µm), c) bottom border of tomato stem scar (1000X,10 µm) , and d) cross section of tomato stem scar (500X, 10 µm).

CHAPTER X

INHIBITION OF BACTERIAL PATHOGENS IN MEDIUM AND SURFACES OF FRESH PRODUCE USING PLANT-DERIVED ANTIMICROBIALS LOADED IN SURFACTANT MICELLES

10.1 Materials and Methods

10.1.1 Preparation of Essential Oil Component Stock Solutions

Stock solutions of 70% (w/v) eugenol (CAS# 97-53-0) (Sigma Aldrich Co., St. Louis, MO) and carvacrol (CAS# 499-75-2) (Sigma Aldrich Co.) were prepared by dissolving each in 95% ethanol (Koptec, King of Prussia, PA). The stock solutions were stored at 5 °C until ready for use.

10.1.2 Encapsulation of EOCs in Surfactant Micelles

Surfynol® 485W (Air Products and Chemical, Inc., Allentown, PA), Tween 20 (Sigma Aldrich Co.), sodium dodecyl sulfate (SDS; Sigma Aldrich Co.), and CytoGUARD® LA 20 (CG20; A&B Ingredients, Fairfield, NJ) (containing 10% [w/w] lauric arginate ester [LAE]) were dispersed in distilled water at room temperature to target concentrations of 1.0, 2.0, 3.5, 5.0, 7.5 and 10.0% (w/v). Eugenol and carvacrol were added to the surfactant solutions to targeted concentrations of 0.01% to 8.0% (w/v) and were stirred until the optical density at 632 nm (OD_{632}) remained constant, indicating complete solubilization *(107)*. The micelle solutions were filter sterilized using a 0.2 or 0.45 μm cellulose acetate membrane filter (VWR International, Radnor, PA) and stored at 5°C for no longer than 36 h prior to assay.

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10.1.3 Maximum Additive Concentration of Antimicrobial Micelles

Samples of loaded micelle solutions (200 µl) were transferred into a 96-well plate and the optical density at 632 nm (OD_{632}) was measured using an Epoch UV/Visible scanning spectrophotometer (Bio-Tek® Instruments, Inc., Winooski, VT). Antimicrobial EOC loading capacity of surfactant micelles was determined by the maximum additive concentration (MAC) *(247).* For each surfactant/oil combination, the MAC was identified as the lowest oil concentration for which an $OD_{632} \geq 0.005$ was recorded after baseline adjustment across triplicate identical replications (n=3) *(107).*

10.1.4 Rheological Analysis of Micelles

To observe the response behavior of micelles following application of shearing, rheological analyses for micelles were performed; analysis was first attempted with a freshly prepared micelle solution of 10% (w/v) CG20 loaded with 0.8% (w/v) eugenol. The creep compliance analysis was performed using Hakke Rheostress 6000 (Thermo Fisher Scientific, Waltham, MA) (cone diameter = 60 mm, cone angel = 1°, cone and plate gap = 0.052 mm). At 10°C, a constant stress at 0.5 Pa and 1.0 Pa (linear viscoelastic region = 0.1 to 1 Pa) was applied to the micelles solution sample for 600 s, and then the stress was removed and the sample was allowed to recover for 600 s. The dynamic oscillatory test was attempted using Hakke Rheostress 6000 (cone diameter = 60 mm, cone angel = 1°, cone and plate gap = 0.052 mm). At 10°C and a shear stress of 0.1 Pa, sinusoidal strain (0.01 to 100 Hz) was applied to the micelles sample. The test was performed for 288 s and 979.6 s. The stress ramp test was performed using Hakke Rheostress 6000 (cone diameter = 60mm, cone angel = 1°, cone and plate gap = 0.052

mm). Shear strain from 0.1 to 100 1/s was applied to a series of micelles-containing samples prepared with 10% (w/v) CG20 + 0.8% eugenol. The test was run at 10°C at a frequency of 0.1 Hz. The ramp up and recovery test was performed using Hakke Rheostress 6000 (cone diameter = 60mm, cone angel = 1°, cone and plate gap = 0.052 mm). Shear strain at 1000 and 5000 1/s was applied to the micelles solution sample for 60 s. After, the shear stress was removed and the viscosity was recorded. The temperature was maintained constant at 5°C for the entire experiment.

10.1.5 Preparation of Bacterial Pathogens for Antimicrobial Assay

E. coli O157:H7 K3999 and *S. enterica* serotype Saintpaul FDA/CFSAN476398 (produce outbreak isolate) were obtained from the Food Microbiology Laboratory culture collection (Department of Animal Science, Texas A&M University, College Station, TX) and maintained on slants of tryptic soy agar (TSA; Becton, Dickinson and Co., Sparks, MD) at 5°C. Working cultures of pathogens were prepared by transferring a loopful of culture from TSA slants to 10 ml tryptic soy broth (TSB; Becton, Dickinson and Co.), and incubating at 35°C for 24 h. After incubation, cultures of pathogens were transferred into sterile 10 ml TSB volumes and incubated at 35°C for 24 h. Genus and species were confirmed biochemically by use of the Vitek® 2 (bioMérieux N.A., Durham, NC). *E. coli* O157:H7 and *S.* Saintpaul serotypes were confirmed by latex agglutination (Remel, Lenexa, KS; *E. coli* O157:H7) or by submission to the U.S. Department of Agriculture National Veterinary Services Laboratory (Ames, IA; *S.* Saintpaul). Pathogens were maintained and handled under biosafety level (BSL) 2

containment at all times according to Texas A&M University Institutional Biosafety Committee policy.

10.1.6 Minimum Inhibitory and Bactericidal Concentrations of EOC Micelles

Overnight culture (24 h) of *E. coli* O157:H7 and *S.* Saintpaul were individually serially diluted in 9.0 ml of 0.1% (w/v) peptone water (PW; Becton, Dickinson and Co.) and then transferred to 9.0 ml double-strength TSB (2X TSB) to a final concentration of approximately 5.0 $\log_{10}$ CFU/ml for use as inocula in minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. For purposes of pathogen inoculum quantification, each pathogen in 2X TSB was serially diluted in 0.1% PW and spread on surfaces of Petri dishes containing sterile TSA. Inoculated TSA dishes were then incubated for 24-48 hr at 35°C prior to colony enumeration.

Two hundred µl of double-strength micelle solutions were added into the first wells of a sterile 96-well plate (Thermo Fisher Scientific, Waltham, MA). One hundred µl of surfactant was added into the remaining wells. One hundred µl of micelle solutions in the first wells were transferred to the second (adjacent) wells and were serially diluted into the remaining wells. Inocula in 2X TSB (100 µl) were lastly added into each well *(187).* Negative controls containing antimicrobial micelles and 2X TSB, but no microorganisms, were included for optical density at 630 nm (OD_{630}) baseline adjustment. Positive controls containing pathogens, ethanol and 2X TSB were also included in the plate. Ethanol was incorporated at the highest calculated concentration that experimental pathogens were exposed to in sample wells containing EOC micelles, to identify the capacity of pathogens to grow in the presence of the alcohol. After plate

preparation, the OD_{630} (0 h) of the test wells was measured. The plates were then incubated at 35°C for 24 h, after which OD_{630} was again recorded. After baseline adjustment, antimicrobial-loaded micelles in which corresponding test wells showed a <0.05 increase in OD_{630} from 0 h to 24 h (ΔOD_{630}) were considered pathogen-inhibitory. The lowest concentrations of antimicrobial oil containing-micelles producing pathogen inhibition across triplicate identical replications (n=3) were identified as the MIC for each EOC/surfactant system *(31)*.

To study the bactericidal activity of micelle solutions after identification of MICs of EOC-containing micelles, 100 µl of solution from pathogen inhibitory wells were spread on TSA-containing Petri plates (1 TSA plate prepared per test well). Inoculated plates were incubated for 24 h at 35°C. The concentrations of micelle solutions producing ≥ 3.0 $\log_{10}$ CFU/ml reduction of the pathogen, defined as plate count of the inoculum prior to MIC assay minus the plate count on the TSA plate for bactericidal assay, were classified as bactericidal. The lowest concentration of EOC micelles over all replications (n=3) were deemed the minimum bactericidal concentration (MBC) *(31)*.

10.1.7 Preliminary Experiments

10.1.7.1 Determination of Application Method and Antimicrobial Activity of EOC Micelles Against Pathogens on Spinach Surfaces

To test the hypothesis that antimicrobial-bearing micelles could reduce pathogen numbers on artificially contaminated produce surfaces, a preliminary experiment was undertaken to determine whether micelle application method would impact observed pathogen reduction on spinach leaf surfaces. Bunched, non-waxed spinach was

purchased from an area grocer, transported immediately to the Food Microbiology Laboratory (Department of Animal Science, Texas A&M University), and washed with sterile distilled water and surface sanitized with 70% ethanol. Sanitized spinach leaves were air-dried in a Biological Cabinet (Class II A/B3) for 60 min prior to preparation for inoculation. After drying, 10 cm^2 samples of spinach were aseptically excised using sterile scalpel and borer, placed in empty sterile Petri dishes, and surface-inoculated with approximately 7.0 $\log_{10}$ CFU/ml of rifampicin-resistant (100 µg/ml; Sigma-Aldrich Co.) cocktailed *E. coli* O157:H7 and *S.* Saintpaul. Rifampicin-resistant mutants (Rif^R) of parent strains were produced using previously published methods *(37).* Pathogen cocktail was applied by spot-inoculation on spinach (adaxial side) of ten spots at 10.0 µl ea. Pathogen-inoculated spinach samples were then air dried at ambient temperature (25±1°C) for 1.0 h to allow pathogen attachment to the produce surface.

After drying, 1.0% SDS + 1.0% eugenol-loaded micelles and 0.125% CG + 0.003125% (calculated delivered concentration following preparation and necessary dilution to achieve twice the MBC for selected micelle systems) eugenol micelle solution and were individually applied to inoculated spinach in Petri dishes by spraying one, two, or three sprays (~1.0 ml per spray) using a sterile misting bottle or by immersing in 20 ml of EOC micelle solution for 2 or 5 min. These concentrations were selected as they provided an EOC of twice the MBC obtained for pathogens from previous experiments. Positive controls (inoculated spinach sample without treatment) and negative controls (uninoculated spinach sample without treatment) were included to determine pathogen attachment to spinach and to confirm no naturally occurring Rif^R

microbes. To enumerate pathogens, a 10 cm^2 sample of inoculated and treated spinach was placed in a stomacher bag and pummeled (230 rpm) with 99 ml of 0.1% peptone diluent for 1 min. Surviving *E. coli* O157:H7 and *S. Saintpaul* were serially diluted in 9 ml of 0.1% peptone diluent and were spread on surfaces of LSPR. The limit of detection for plating assays was 100 CFU/10 cm^2. Following 24 h incubation at 35°C, colonies of Rif^R *E. coli* O157:H7 (white colonies with yellow haloes indicating ability to utilize lactose without sulfite reduction) and *S.* Saintpaul (black-centered colonies surrounded by a pink halo indicated lack of lactose utilization with sulfite reduction) were counted. The assay was performed in triplicate (n=3).

10.1.8 Statistical Analyses

All experiments were replicated thrice identically. Logarithmically transformed (base 10) counts of pathogens were analyzed for differences between treatments by analysis of variance (ANOVA) at $\alpha=0.05$ via JMP v10.0.0 (SAS Institute Inc., Cary, NC, USA). Significantly differing mean $\log_{10}$-transformed pathogen counts were separated by Student's t-test ($p<0.05$).

10.2 Results and Discussion

10.2.1 Maximum Additive Concentrations of EOC-Loaded Micelles

During micelles preparation, baseline-adjusted OD_{632} remained zero as EOC droplets were encapsulated in micelles. OD_{632} increased from zero when the concentrations of EOC exceeded the MAC at a specific surfactant concentration, indicating that oil droplets could not be completely solubilized/emulsified in available surfactant micelles *(109)*. MACs of eugenol and carvacrol increased with increasing

surfactant concentrations (Figure 10-1). Excepting SDS, MACs of eugenol for all surfactants were higher than MACs of carvacrol at each surfactant concentration. The highest MACs of both EOCs were observed when SDS was used for encapsulation of eugenol or carvacrol (5.5%) while the lowest MAC was observed in Tween 20 + carvacrol (0.7%) (Figure 10-1). Overall, for carvacrol, surfactants followed the trend from least to greatest loading of Tween 20<CG20<Surfynol® 485W<SDS, whereas for eugenol CG20<Tween 20<Surfynol® 485W<SDS.

Factors affecting MACs of micelles are reported to include the nature of surfactant (e.g. increased MAC of hydrophobic compound residing in the micelle core with increasing alkyl chain length up to C_{16}), nature of hydrophobic compound, environmental temperature, and the hydrophile-lipophile balance (HLB) of surfactant, etc. *(107, 248)*. Among the surfactants used in this study, the highest loading/encapsulation capacity, observed with SDS, may have been due to the low aggregation number (~50), the number of surfactant molecules required to form one micelle, which resulted in more micelles at a specific surfactant concentration *(14, 104)*.

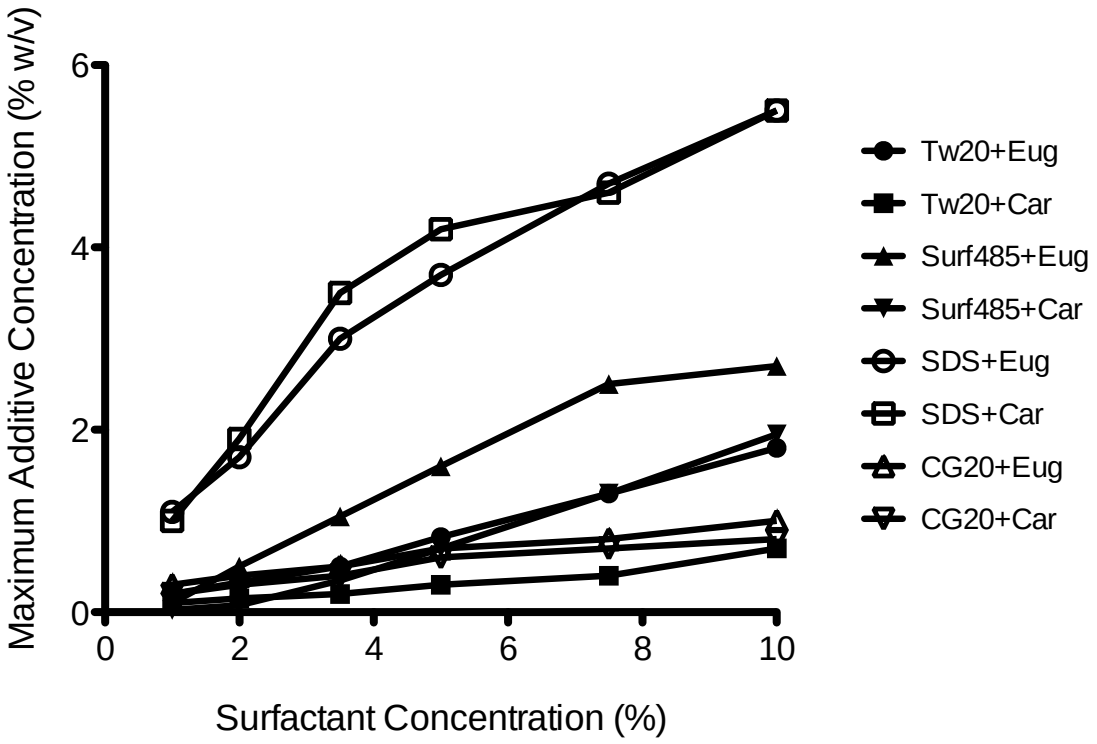

FIGURE 10-1. Maximum additive concentrations of antimicrobial essential oil component (EOC)-bearing surfactant micelles. Values indicate the lowest EOC concentration (% w/v) for which optical density at 632 nm (OD632) > 0.05 for each of three identical replications (n=3). Tw20:Tween 20; Surf485: Surfynol. 485W; SDS: sodium dodecyl sulfate; CG20: CytoGuard. LA20. Reprinted with permission from Ruengvisesh, S., A. Loquercio, E. Castell-Perez, and T. M. Taylor. 2015. Inhibition of Bacterial Pathogens in Medium and on Spinach Leaf Surfaces using Plant-Derived Antimicrobials Loaded in Surfactant Micelles. *J Food Sci*. 80:M2522-9 *(200).*

(104). Conversely, the higher aggregation number of Tween 20 (~86) may have contributed to the low observed encapsulation capacity *(28)*. Encapsulation of eugenol and carvacrol in surfactants (Surfynol® 485W and 465) were previously reported by Gaysinsky and others *(108)*. In their study, MACs increased with increasing surfactant concentrations;Surfynol surfactants, MACs of eugenol were higher than of carvacrol *(107)*. Ariyaprakai and Dungan *(8)* studied solubilization of n-hexadecane and n-tetradecane in different concentrations of SDS and Tween 20; at most surfactant concentrations, MACs of oils in SDS were higher than in Tween 20. The possible solubilization mechanisms of hydrophobic compounds in surfactants can be: 1) direct oil solubilization in aqueous solution followed by incorporation into micelles in the aqueous phase; 2) uptake of oil by empty micelles due to collision of micelles with the emulsion droplet surface, and; 3) spontaneous "budding-off" of oil and surfactant molecules from a droplet surface to form micelles *(159)*. Overall, data indicate that SDS micelles were optimal for EOC loading, and thus for delivery of antimicrobial EOCs to foodborne pathogens.

10.2.2 Rheological Characteristics of EOC-Containing Micelles

In this study, rheological analyses were attempted for antimicrobial micelles, beginning with 10% CG20 micelles containing 0.8% eugenol. However, reasonable data could not be obtained from creep compliance analysis, dynamic oscillatory analysis, and ramp up and recovery testing (data not shown). This may have been due to inadequate sensitivities of the cone and plate used, which resulted in inability to detect responses from dilute micelles solution. Nevertheless, shear stress (Pa) analysis and shear rate (s^{-1})

indicated Newtonian behavior of 10% CG20 + 0.8% eugenol micelles, indicating that solution viscosity did not change as a function of shear rate, a result similar to others' analysis of rheological properties of EOC-bearing nano-emulsions *(261)* (Figure 10-2). Further rheological evaluation of other surfactant/EO combinations was therefore not pursued.

10.2.3 MICs and MBCs of EOC in Surfactant Micelles Against Foodborne Pathogens

MIC and MBC values of EOC-loaded micelles against *E. coli* O157:H7 were similar to those for *S.* Saintpaul (Table 10-1). For *E. coli* O157:H7 and *S.* Saintpaul, the lowest MIC observed was 0.5% SDS + 0.113% carvacrol while the highest MIC was 20% Tween 20 + 2% carvacrol. The lowest and highest MBCs against the two pathogens were 0.0625% CG20 + 0.00156% eugenol or carvacrol and 25% Tween 20 + 4.5% carvacrol, respectively. The MIC of CG20 + eugenol or carvacrol against *E. coli* O157:H7 and *S.* Saintpaul could not be obtained since the highest non-inhibitory concentration of CG20 (0.007813%) and the MACs of eugenol and carvacrol (0.0125%) did not show inhibitory effects against either pathogen. For all systems, reported concentrations indicate the final concentrations of surfactant and encapsulated EOC applied to pathogens in the experimental well (Table 10-1). Due to the low MAC of carvacrol Tween 20, higher concentrations of Tween 20 micelles were likely required

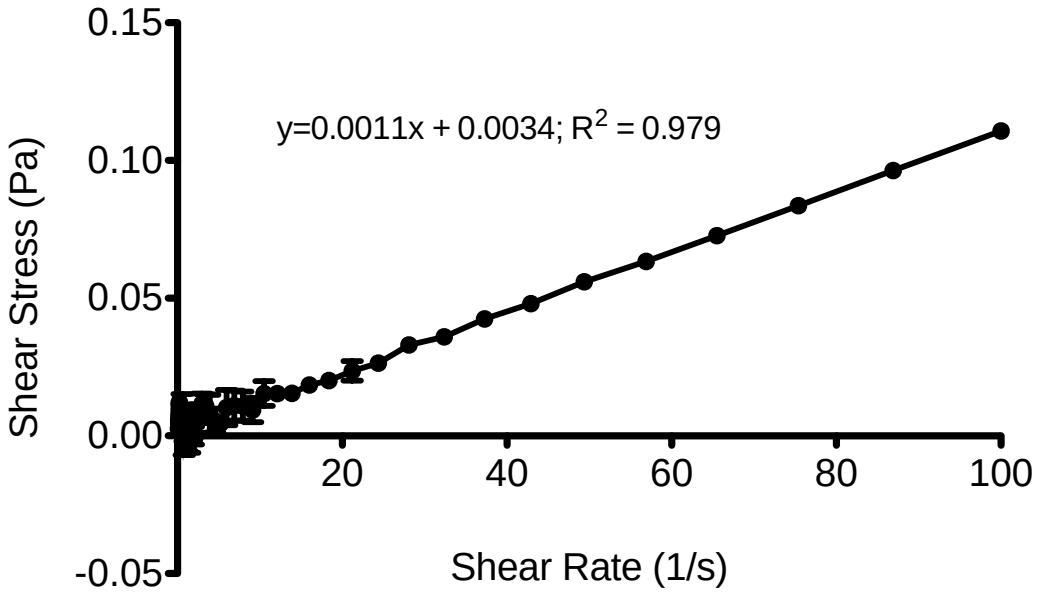

FIGURE 10-2. Shear stress response of antimicrobial-bearing micelles to increased shearing. Values depict means of triplicate identical replications (n=3), with error bars depicting one sample standard deviation from the mean. Equation and R^2 give linear equation of best-fit line and correlation coefficient, respectively. Micelles were constructed of 20.0% (w/v) CytoGuard® LA 20 + 0.8% (w/v) eugenol. Reprinted with permission from Ruengvisesh, S., A. Loquercio, E. Castell-Perez, and T. M. Taylor. 2015. Inhibition of Bacterial Pathogens in Medium and on Spinach Leaf Surfaces using Plant-Derived Antimicrobials Loaded in Surfactant Micelles. *J Food Sci*. 80:M2522-9 *(200)*.

TABLE 10-1: Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) of surfactant + essential oil component (EOC) micelles against foodborne pathogens.[a]

Surfactant (% w/v) + EO (% w/v)[c]	***Escherichia coli* O157:H7**		***Salmonella Saintpaul***	
	MIC (Surfactant + EO)	**MBC (Surfactant + EO)**	**MIC (Surfactant + EO)**	**MIC (Surfactant + EO)**
Tween 20 + Eugenol	2.0 + 0.325	7.5 + 1.3	Tween 20 + Eugenol	2.0 + 0.325
Surfynol 485W+ Eugenol	2.0 + 0.2	2.0 + 0.4	Surfynol 485W+ Eugenol	2.0 + 0.2
SDS + Eugenol	0.5 + 0.25	0.5 + 0.5	SDS + Eugenol	0.5 + 0.25
CG20 + Eugenol	ND[b]	0.0625 + 0.00156	CG20 + Eugenol	ND[b]
Tween 20 + Carvacrol	20.0 + 2.0	25.0 + 4.5	Tween 20 + Carvacrol	20.0 + 2.0
Surfynol 485W + Carvacrol	3.5 + 0.3	7.5 + 1.2	Surfynol 485W + Carvacrol	3.5 + 0.3
SDS + Carvacrol	0.5 + 0.113	0.5 + 0.225	SDS + Carvacrol	0.5 + 0.113
CG20 + Carvacrol	ND	0.0625 + 0.00156	CG20 + Carvacrol	ND

[a]Values depict the lowest concentrations of surfactant and EO micelles consistently producing inhibition or inactivation of indicated pathogen across triplicate identical replications, where inhibition was defined as ΔOD_{630} from 0 to 24 hr at 35°C was <0.05, and inactivation was defined as achieving $\geq$3.0 $\log_{10}$ CFU/ml reduction in pathogen numbers, determined as the difference from plate count of inoculated pathogen cells minus the plate count of pathogen colonies following spreading of 0.1 ml from inhibitory concentration wells on the surface of tryptic soy agar and subsequent 24 hr incubation at 35°C prior to colony enumeration and $\log_{10}$-transformation.

[b]ND: Not determined. The highest non-inhibitory concentration of CG20 at the maximum loading for both oils (0.0125% w/v) was not observed to produce inhibition of pathogens.

[c]SDS: sodium dodecyl sulfate; CG20: CytoGuard LA 20.

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to sufficiently encapsulate carvacrol to produce inhibitory or bactericidal activity against pathogens.

Many studies have shown the antimicrobial efficacy of nano-encapsulated plant-derived EOCs. Gaysinsky and others *(107)* reported MICs of carvacrol and eugenol ranged from 0.02 to 1.25% in 0.5% Surfynol 485W or Surfynol 465 micelles for *E. coli* O157:H7 and *L. monocytogenes.* Entrapment of various plant oils in alginate systems produced MICs of mesophilic bacteria on produce surfaces of 0.5-0.7% *(193)*. Donsì and others *(76)* reported MICs and MBCs of a terpene mixture and limonene encapsulated in nano-emulsion against *Lactobacillus delbrueckii*, *Saccharomyces cerevisiae* and *E. coli* ranged from 5.0 to >25.0 g/l. Mechanisms of action of EOCs against microorganisms are reported to include cytoplasmic membrane disruption, destabilization of proton motive force, disturbed electron flow, active transport, coagulation of the cell content, as well as suppression of gene expression of various pathogenesis elements *(137, 146, 157, 212, 235, 236)*. Both hydrophilic and hydrophobic fractions of EOCs are involved in antimicrobial activity *(208)*. Hydrophilic components of EOCs (e.g. hydroxyl group) interact with polar components (e.g. outer membrane proteins) of the bacterial cell membrane while hydrophobic components react with lipids on the membrane and result in increased membrane permeability, disturbed enzyme systems and genetic material *(208, 211, 222)*. It has been reported that Gram-negative bacteria are more tolerant to the antimicrobial activity of differing EOCs, possibly a function of the presence of an outer membrane and lipopolysaccharide that screen hydrophobic molecules and limit their access to the periplasm or cytoplasm *(36, 68, 199, 222, 227)*.

The roles of surfactant micelles in antimicrobial delivery to pathogens can include: 1) enhanced dispersion of EOCs in aqueous solution; 2) transport of EOCs to microbial membranes, and; 3) disruption of microbial membranes to enhance payload uptake *(7, 79, 110, 128, 159, 224)*. Some surfactants, including SDS, have been reported to denature membrane-located proteins and damage the membranes of microbial cells, leading to leakage of cytoplasmic contents and potentially depolarization of the membrane *(252, 259, 260)*. Ionic and nonionic surfactants were used in this research. Surfynol® 485W is a nonionic surfactant containing an acetylenic group and two polyoxyethylene groups *(109)*. It has low dynamic surface tension as well as stability in high ionic strength conditions *(183)*. Tween 20 is a nonionic surfactant composed of fatty acid esters of polyoxyethylene sorbitan monolaurate and is widely used as a stabilizer in food and pharmaceutical industries *(142)*. SDS is an anionic surfactant approved as an emulsifier in or with egg whites, a whipping agent, and a wetting agent *(89)*. CG20 contains 10% LAE, a cationic amino acid-based surfactant that is a derivative of lauric acid, L-arginine and ethanol *(11, 155)*. LAE has been approved by the FDA *(88)* as a generally recognized as safe (GRAS) antimicrobial ingredient for multiple food applications. In this study, the low MBCs of EOCs encapsulated in SDS and CG20 against pathogens were likely due in part to the antimicrobial effects of SDS and CG20 *(22, 218)*. SDS has been reported to denature protein and damage cell membranes of microorganisms *(102, 252, 266)*. Since the net charge of bacterial membrane is negative, the lowest MBCs for CG20 micelles against the pathogens might have been enhanced by the cationic charge of LAE that caused electrostatic attraction

between micelle particles and microbial membranes *(71, 211, 247)*. In this study, EOC-loaded micelles demonstrated antimicrobial activity against foodborne pathogens and thus might be a promising pathogen intervention method in food systems. Since micelles are thermodynamically favored with respect to their formation in systems containing hydrophobic compounds dispersed in aqueous solvent, they may remain kinetically stable for an extended period of time if the initial conditions are unchanged *(163, 198)*. However, in practice, changes in environmental conditions (e.g. pH, ionic strength, temperature, etc.) do occur and result in loss of stability (e.g. Ostwald ripening, flocculation, aggregation, coalescence) of micelles in the system *(163)*. Thus further research in EOC micelle stability and payload release in food systems is still required to elaborate their utility for pathogen inhibition in food systems.

10.2.4 Determination of Application Methods for Antimicrobial-Loaded Micelles on Spinach (Preliminary Experiment)

Populations of *S.* Saintpaul after treatment (one, two, or three sprays, 2 or 5 min immersion) with 1% SDS + 1% eugenol micelle solution or 0.125% CG20+0.003125% eugenol (predicted concentration based upon dilution scheme from MIC assay) micelle solution are shown in Figure 10-3. The mean population of *S.* Saintpaul on untreated spinach samples (control) was 6.7±0.3 $\log_{10}$ CFU/cm^2. The population of *S.* Saintpaul after treatment with EOC-loaded micelles ranged from 2.2 to 4.9 $\log_{10}$ CFU/cm^2. Except for the 2-spray application of 0.125% CG20+0.003125% eugenol micelle, all treatments of EOC-loaded micelles yielded lower *S.* Saintpaul populations compared to the control ($p<0.05$). The antimicrobial effect of two sprays CG20+eugenol micelles on *S.* Saintpaul

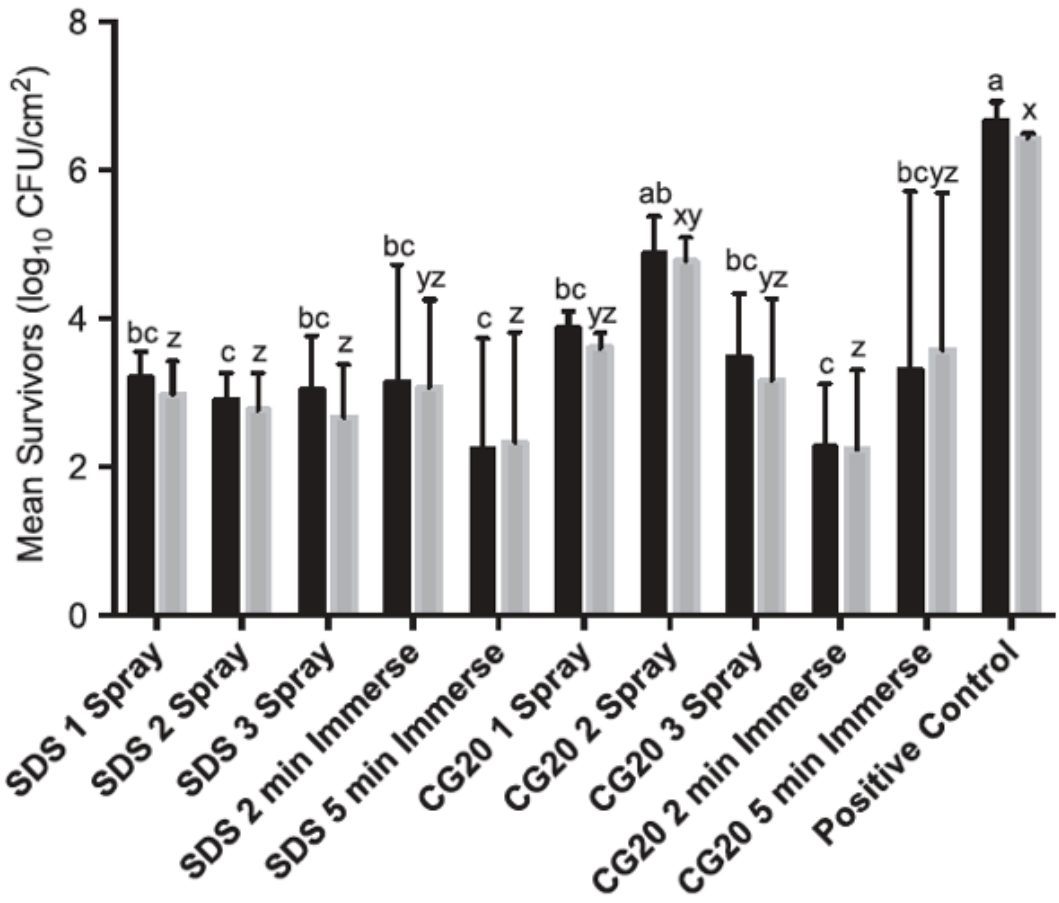

FIGURE 10-3. Survival of bacterial pathogen numbers on spinach treated with eugenol-containing micelles applied by spray or immersion. Columns (black: *Salmonella* Saintpaul; gray: *Escherichia coli* O157:H7) depict means from triplicate identical replications (n = 3) while error bars depict standard deviation from the mean. SDS, sodium dodecyl sulfate; CG20, CytoGuard®LA 20. Micelles were applied by 1, 2, or 3 spray, or by 2- or 5-min immersion in 20 mL sterile distilled water; micelles contained 1.0% SDS + 1.0% eugenol or 0.125% CG20 + 0.003125% eugenol. Within pathogen, means not connected by same letter are significantly different ($p<0.05$). Reprinted with permission from Ruengvisesh, S., A. Loquercio, E. Castell-Perez, and T. M. Taylor. 2015. Inhibition of Bacterial Pathogens in Medium and on Spinach Leaf Surfaces using Plant-Derived Antimicrobials Loaded in Surfactant Micelles. *J Food Sci*. 80:M2522-9 *(200)*.

survival did not differ from that of one spray, three spray, or 5 min immersion of CG20+eugenol micelles, or from one spray, three spray, or 2 min immersion of SDS+eugenol micelles. However, treatment with two sprays of CG20+eugenol micelles resulted in greater *S.* Saintpaul survival versus treatment with two sprays or 5 min immersion application of SDS+eugenol micelles and 2 min immersion CG20+eugenol micelles.

Figure 10-3 depicts the survival of *E. coli* O157:H7 after spray or immersion treatment with eugenol-loaded 1% SDS or 0.125% CG micelle systems. The population of *E. coli* O157:H7 on the control spinach sample was 6.4±0.1 $\log_{10}$ CFU/cm^2. Treatments with EOC-loaded micelles resulted in *E. coli* O157:H7 populations ranging from 2.2 to 4.8 $\log_{10}$ CFU/cm^2. Excepting two spray application of CG20+eugenol micelles, all EOC-loaded micelle treatments produced significant reductions in *E. coli* O157:H7 numbers on spinach ($p<0.05$). Antimicrobial effects of CG20+eugenol micelles applied via two sprays were not different from CG20 micelles applied via one or three sprays, or by 5 min immersion application (Figure10-3). Nevertheless, *E. coli* O157:H7 populations on spinach after treatment with two sprays of CG20+eugenol micelles were greater than those treated by one, two, or three sprays, and 5 min immersion application of eugenol-loaded SDS micelles. According to results obtained from MIC assays, EOCs loaded in CG20 and SDS micelles effectively inhibited pathogen growth in liquid medium. It has been reported that higher antimicrobial concentrations are typically required to obtain similar antimicrobial effects as those obtained *in vitro (20, 42, 207)*. Thus, twice the MBCs of eugenol loaded into CG20 and

SDS micelles were used in this experiment to reduce numbers of inoculated pathogens on spinach surfaces. Results showed that while CG20 micelles applied to spinach by two sprays produced the lowest antimicrobial effect, other treatment methods were similarly effective in reducing *S.* Saintpaul and *E. coli* O157:H7 on spinach ($p \geq 0.05$) (Figure 10-3). Since spraying may have utilized a reduced application volume of micelles, it may represent a useful method for applying antimicrobial-bearing micelles onto fresh produce. Ponce and others *(190)* reported that, while spraying of plant oils produced low antimicrobial utility for reducing pathogens on lettuce leaves, spraying of essential oil components resulted in the lowest degree of lettuce sensorial acceptability loss. Results gathered in the present study suggest that micelle loading of EOCs may produce multi-$\log_{10}$-cycle reductions of pathogens on produce surfaces without necessarily harming produce palatability and consumer acceptance. Quantitative organoleptic characterization of treated spinach was not completed as a part of the current study. Nonetheless, visual observation of treated spinach leaves indicated no detectable change in leaf color within the experimental incubation period prior to processing for microbiological analysis (Data not shown).

On surfaces of fresh produce, cracks, pockets, crevices and native openings (e.g., stomata) may provide protection to cross-contaminating microorganisms from various food safety interventions, including plant-derived antimicrobials *(263).* The efficacy of antimicrobial or sanitizer application to produce surfaces may be improved when antimicrobials or sanitizing compounds are granted enhanced access to microorganisms residing in protected sites *(263).* The entrapment of antimicrobial hydrophobic oils into

surfactant micelles may assist the delivery of antimicrobial oils to pathogens on produce surfaces due to surfactant-driven modulation of produce surface hydrophobicity, thereby improving their pathogen decontamination capacity *(261)*. Additionally, micelle entrapment has been suggested to enhance the dispersion of plant-derived EOCs in aqueous systems, such as wash or flume waters encountered during produce packing. It is expected that emulsified EOCs would exert antimicrobial effects against suspended microbial pathogens given successful contact of micelle with pathogen cell *(224)*. Finally, while comprehensive comparison of un-encapsulated versus encapsulated eugenol was not completed in this study, previous research has reported that encapsulation of EOCs reduces their volatilization rate and improves their delivery to foods via aqueous carrier fluids *(188, 189)*.

CHAPTER XI

INHIBITION OF BACTERIAL PATHOGENS ON SURFACES OF FRESH PRODUCE USING PLANT-DERIVED ANTIMICROBIALS LOADED IN SURFACTANT MICELLES AND OTHER ANTIMICROBIALS

11.1 Materials and Methods

11.1.1 Preliminary Experiment

11.1.1.1 Determination of Priming Agent Efficacy and Antimicrobial Activity of EOC Micelles Against Pathogens on Spinach Surfaces

To test the hypothesis that priming could improve access to microorganisms residing in protected sites (e.g. cracks and crevices), a preliminary experiment was performed to determine whether utilizing priming agents prior to antimicrobial application of fresh produce surfaces would improve pathogen reduction on spinach leaf surfaces. Bunched, non-waxed spinach was purchased from a local grocer, transported immediately to the Food Microbiology Laboratory (Department of Animal Science, Texas A&M University), and washed with sterile distilled water and surface sanitized with 70% ethanol. Sanitized spinach leaves were air-dried in a Biological Cabinet (Class II A/B3) for 60 min prior to preparation for inoculation. After drying, two pieces of 10 cm^2 of spinach were aseptically excised using sterile scalpel and borer, placed in empty sterile Petri dishes, and surface-inoculated with approximately 7.0 $\log_{10}$ CFU/ml of cocktailed Rif^R *E. coli* O157:H7 and *S.* Saintpaul. Pathogen cocktail was applied by spot-inoculation on spinach (adaxial side) of ten spots at 10.0 µl ea. Pathogen-inoculated spinach samples were then air dried at ambient temperature (25±1°C) for 1.0 h to allow

pathogen attachment to the produce surface. After drying, one, two, or three sprays (~1.0 ml per spray) of a priming agent (70% EtOH) were applied on surfaces of spinach samples. Following application of a priming agent, 1.0% SDS + 1.0% eugenol-loaded was individually applied to inoculated spinach in Petri dishes using a sterile misting bottle (1 spray or 2 sprays) or by immersing in 20 ml of EOC micelle solution for 2 min. Positive controls (inoculated spinach sample without treatment) and negative controls (uninoculated spinach sample without treatment) were included to determine pathogen attachment to spinach and to confirm no naturally occurring Rif^R microbes. To enumerate pathogens, two pieces of 10 cm^2 sample of inoculated and treated spinach were placed in a stomacher bag and pummeled (230 rpm) with 99 ml of 0.1% peptone diluent for 1 min. Surviving *E. coli* O157:H7 and *S.* Saintpaul were serially diluted in 9 ml of 0.1% peptone diluent and were spread on surfaces of LSPR. The limit of detection for plating assays was 100 CFU/20 cm^2. Following 24 h incubation at 35°C, colonies of Rif^R *E. coli* O157:H7 and *S.* Saintpaul were counted. The assay was done in triplicate (n=3).

11.1.1.2 Determination of EOCs Efficacy

A preliminary experiment was done to test the efficacy of eugenol-encapsulated micelles versus carvacrol-encapsulated micelles for inactivating pathogens on tomato samples. Non-waxed tomatoes were purchased from a local grocer, transported immediately to the Food Microbiology Laboratory (Department of Animal Science, Texas A&M University), and washed with sterile distilled water and surface sanitized with 70% ethanol. Sanitized spinach leaves were air-dried in a Biological Cabinet (Class

II A/B3) for 60 min prior to preparation for inoculation. After drying, two pieces of 10 cm^2 of spinach were aseptically excised using sterile scalpel and borer, placed in empty sterile Petri dishes, and surface-inoculated with approximately 7.0 log_{10} CFU/ml of cocktailed Rif^R *E. coli* O157:H7 and *S.* Saintpaul. Pathogen cocktail was applied by spot-inoculation on spinach of ten spots at 10.0 µl ea. Pathogen-inoculated spinach samples were then air dried at ambient temperature (25±1°C) for 1.0 h to allow pathogen attachment to the produce surface.

After drying, 1.0% SDS + 1.0% eugenol-loaded or 1.0% SDS + 0.9% carvacrol-loaded micelles was individually applied to inoculated tomato samples in Petri dishes by immersing in 20 ml of EOC micelle solution for 2 min. Positive controls (inoculated tomato sample without treatment) and negative controls (uninoculated tomato sample without treatment) were included to determine pathogen attachment to tomatoes and to confirm no naturally occurring Rif^R microbes. Treated samples were stored for 0 and 2 days at 5°C prior to pathogen enumeration. To enumerate pathogens, samples were placed in a stomacher bag and pummeled (230 rpm) with 99 ml of 0.1% peptone diluent for 1 min. Surviving *E. coli* O157:H7 and *S.* Saintpaul were serially diluted in 9 ml of 0.1% peptone diluent and were spread on surfaces of LSPR. The limit of detection for plating assays was 100 CFU/30 cm^2. Following 24 h incubation at 35°C, colonies of Rif^R *E. coli* O157:H7 and *S.* Saintpaul were counted. The assay was performed in triplicate (n=3).

11.1.2 Antimicrobial Activity of Eugenol-Loaded Micelles and Other Antimicrobial Agents against Pathogens and Natural Microbiota on Tomato and Spinach

Non-waxed, unwashed vine tomatoes and spinach were purchased from a local grocer, transported immediately to the Food Microbiology Laboratory (Department of Animal Science, Texas A&M University), Three pieces of 10 cm^2 of spinach and tomato were aseptically excised using sterile scalpel and borer, placed in empty sterile Petri dishes, and surface-inoculated with approximately 7.0 $\log_{10}$ CFU/ml of cocktailed Rif^R *E. coli* O157:H7 and *S.* Saintpaul. Pathogen cocktail was spot-inoculated on samples (ten spots at 10.0 µl). Pathogen-inoculated spinach samples were then air dried at ambient temperature (25±1°C) for 1.0 h to allow pathogen attachment to the produce surface.

To test the pathogen inhibitory effect of each treatment, after drying, encapsulated eugenol (1.0% SDS + 1.0% eugenol-loaded micelles), free eugenol (1.0% eugenol), empty micelles (1.0% SDS), 200 ppm chlorine pH 7.0, and sterile distilled water were individually applied to inoculated spinach and tomato samples in Petri dishes by immersing in 20 ml of the treatment solution. Encapsulated eugenol was prepared as described in 10.1.2. Free eugenol, empty micelles and chlorine solutions were prepared by adding eugenol, SDS and chlorine individually to sterile distilled water to obtain the desired concentrations and were stirred using a magnetic stirrer. Chlorine solution was pH-adjusted using 0.1N HCl. Positive controls (inoculated sample without treatment) and negative controls (uninoculated sample without treatment) were included to determine pathogen attachment to tomatoes and to confirm no naturally occurring Rif^R microbes. To test the inhibitory effect of treatments against produce natural microbiota,

encapsulated eugenol, free eugenol, empty micelles, chlorine, and sterile distilled water were individually applied to Petri dishes via 2 min immersion in 20 ml of treatment solution. Positive controls (uninoculated sample without treatment) were included to determine the initial numbers of natural microbiota on the produce samples prior to treatment. On day 0 of storage, after treatments, samples were immediately processed for microbial enumeration. Other samples were initially stored at 5°C and were withdrawn on day 3, 5, 7, and 10 of storage for microbial enumeration. At day 5, one set (set B) of samples were transitioned to 15°C to simulate a postharvest temperature abuse condition while the other set (set A) remained at 5°C. To enumerate pathogens, samples were placed in stomacher bags and pummeled (230 rpm) with 99 ml of 0.1% peptone diluent for 1 min. Surviving pathogens were serially diluted in 9 ml of 0.1% peptone diluent and were spread on surfaces of LSPR. The limit of detection for plating assays was 100 CFU/30 cm^2. Following 24 h incubation at 35°C, colonies of Rif^R *E. coli* O157:H7 and *S.* Saintpaul were counted. For enumeration of native microbiota (aerobic bacteria, Enterobacteriaceae, and yeasts and molds), resulting samples were serially diluted in 9 ml of 0.1% peptone diluent and were spread on 3M™ Petrifilm™ Aeobic Count Plates, 3M™ Petrifilm™ Enterobacteriaceae Count Plates, and 3M™ Petrifilm™ Yeast and Mold Count Plates. Petrifilm™ Aeobic, and 3M™ Petrifilm™ Enterobacteriaceae Count Plates were incubated at 35°C for 48 hr while 3M™ Petrifilm™ Yeast and Mold Count Plates were incubated at 25°C for 5 days. Colonies were counted after incubation. The assay was performed in triplicate with duplicate samples for each replicate (n=6).

11.1.3 Z-Average Measurement

Measurements of the z-average particle diameter (mean hydrodynamic diameter) and polydispersity index (an estimate of the width of the distribution) of encapsulated eugenol (1.0% SDS + 1.0% eugenol-loaded micelles), free eugenol (1.0% eugenol), and empty micelles (1% SDS) were conducted using a dynamic light scattering technique (Zetasizer Nano-ZS90, Malvern Instruments, Southborough, MA) at 90° scattering angle. The technique measures the time-dependent fluctuations in the intensity of scattered light from a suspension of particles that that undergoes Brownian motion. Intensity fluctuation allows for the determination of the diffusion coefficients that yield the particle size through the Stokes-Einstein equation. All measurements was conducted in triplicate (n=3) at 25°C.

11.1.4 ζ-Potential Measurement

The ζ-potential of encapsulated eugenol (1.0% SDS + 1.0% eugenol-loaded micelles), free eugenol (1.0% eugenol), and 1% SDS (empty micelles) were determined by measuring the direction and velocity that particles moved in the applied electric field of a particle electrophoresis instrument (Malvern Instruments). All measurements was conducted in triplicate (n=3) at 25°C.

11.1.5 Statistical Analyses

All experiments were replicated thrice identically. Logarithmically transformed (base 10) counts of pathogens obtained following treatments on produce samples, ζ-potential, and z-average were analyzed for differences between treatments by analysis of variance (ANOVA) at α=0.05 via JMP v10.0.0 (SAS Institute Inc., Cary, NC, USA).

Significantly differing mean $\log_{10}$-transformed pathogen counts, ζ-potential, and z-average were separated by Student's t-test ($p<0.05$). For experiment 11.1.2, logarithmically transformed (base 10) counts for each pathogen were compared within storage day for each sample set, over 10-day storage period for each sample set, and over 10-day storage period across both sample sets by ANOVA at $\alpha=0.05$ via JMP v10.0.0. Significantly differing mean $\log_{10}$-transformed pathogen counts, were separated by Student's t-test ($p<0.05$).

11.2 Results and Discussion

11.2.1 Determination of Priming Agent Efficacy in Combination with EOC Micelles Against Pathogens on Spinach Surfaces (Preliminary Experiment)

Ethanol (70%) was used as a priming agent prior to EOC micelles application in this study. Due to its low surface tension (35.51 mN/m) *(241),* 70% EtOH spreads well and also may serve as a wetting agent *(219)*. Thus, it was hypothesized that 70% ethanol could access cracks, crevices, and also minimize air pockets on spinach surfaces, resulting in improved contact between spinach surfaces and EOC antimicrobial micelles. For *S.* Saintpaul, priming with 70% ethanol (1 and 2 sprays) followed by application of eugenol-loaded micelles by spraying (1 and 3 sprays) or 2 min immersion yielded 1.9 to 3.9 $\log_{10}$ reduction (Figure 11-1). Without priming with 70% ethanol, treatments with eugenol-loaded micelles alone by spraying or immersion resulted in 2.1 to 2.7 $\log_{10}$ reduction. For *E. coli* O157:H7, priming with 70% ethanol (1 and 2 sprays) followed by application of eugenol-loaded micelles by spraying (1 and 3 sprays) or 2 min immersion resulted in 1.6 to 3.3 $\log_{10}$ reduction (Figure 11-2). Treatments with eugenol-loaded

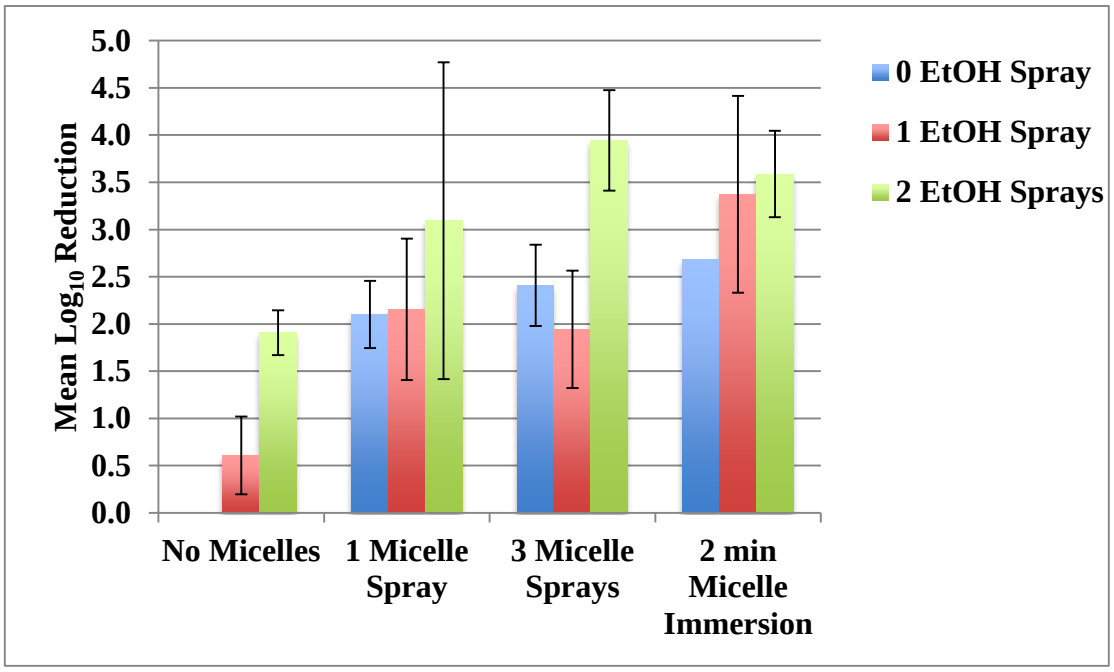

FIGURE 11-1. Log_{10} reduction of *Salmonella* Saintpaul on spinach treated with eugenol-containing micelles applied by spray or immersion. Columns depict means log_{10} reduction from triplicate identical replications (n = 3) while error bars depict standard deviation from the mean. The limit of detection is 0.7 log_{10} CFU/cm^2.

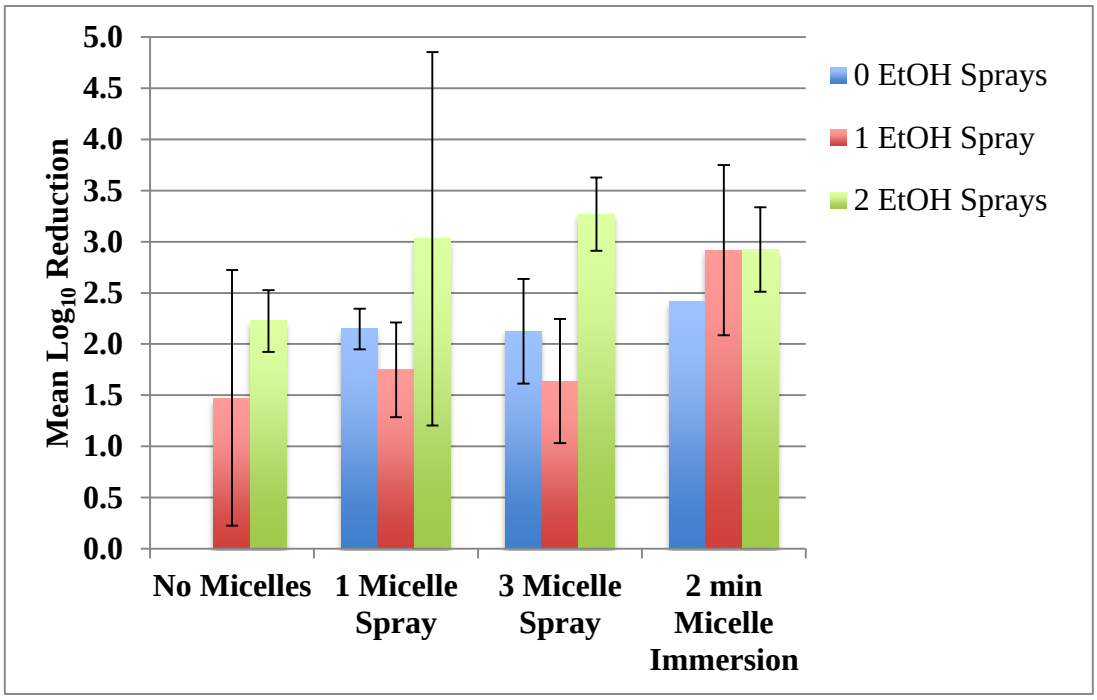

FIGURE 11-2. Log_{10} reduction of *Escherichia coli* O157:H7 on spinach treated with eugenol-containing micelles applied by spray or immersion. Columns depict means log_{10} reduction from triplicate identical replications (n = 3) while error bars depict standard deviation from the mean. The limit of detection is 0.7 log_{10} CFU/cm^2.

micelles alone without 70% ethanol priming yielded 2.1 to 2.4 $\log_{10}$ reduction. No significant difference among the methods of eugenol-loaded micelle application was observed ($p \geq 0.05$). Nevertheless, the effect of priming with 70% ethanol was significant ($P < 0.05$); 2 sprays of 70% ethanol resulted in greater reductions of pathogens than 0 and 1 sprays 70% ethanol. The combined effect of ethanol priming and eugenol-load micelle application, however, did not differ among treatments ($p \geq 0.05$). Overall, nanoencapsulated eugenol micelles in combination with a surface tension reducing agent had the ability to reduce pathogen level on the surfaces of spinach significantly more than nanoencapsulated eugenol micelles applied singly.

11.2.2 Determination of EOC Efficacy against Pathogens on Tomato Surfaces (Preliminary Experiment)

This preliminary was conducted to determine the efficacy of free EOCs (0.9% carvacrol, and 1% eugenol) versus encapsulated EOCs (1% SDS + 0.9% carvacrol, and 1% SDS + 1% eugenol). Inoculated tomato samples were individually treated with encapsulated eugenol, free eugenol, encapsulated carvacrol, and free carvacrol and were stored at 5 °C for 0 day and 2 days. All treatments reduced populations of *S.* Saintpaul to undetectable level (0.4 $\log_{10}$ CFU/cm^2 was assigned for statistical analysis) in both tomato sample sets stored at 5°C for 0 day and 2 days (Figure 11-3 and Figure 11-4). For day 0, populations of *E. coli* O157:H7 after treatment with encapsulated eugenol, free eugenol, encapsulated carvacrol, and free carvacrol were 1.0, 1.0, 0.4, and 0.4 $\log_{10}$ CFU/cm^2 respectively (Figure 10-6). For day 2 samples, encapsulated eugenol, free eugenol, encapsulated carvacrol, and free carvacrol treatment resulted in 0.4, 1.3, 0.4,

and 0.4 $\log_{10}$ CFU/cm^2 of *E. coli* O157:H7 populations respectively (Figure 10-7); population of *E. coli* O157:H7 after free eugenol treatment was significantly higher than those from other treatments ($p<0.05$). For day 0 and day 2 samples, all treatments yielded lower populations of *S.* Saintpaul and *E. coli* O157:H7 compared to controls ($p<0.05$). Since significantly different antimicrobial effect between encapsulated eugenol and non-encapsulated eugenol was observed, eugenol was chosen for encapsulation in surfactant micelles for further pathogen inhibition assays on fresh produce.

11.2.3 Eugenol-Loaded Micelles and Other Treatments against Pathogens and Natural Microbiota on Tomato and Spinach

11.2.3.1 Tomato

In this study, encapsulated eugenol (1% SDS + 1% eugenol), free eugenol (1% eugenol), empty micelles (1% SDS), 200 ppm chlorine, and sterile distilled water were individually applied to pathogen-inoculated and uninoculated tomato samples via immersion to determine the efficacy of treatments for inactivation of pathogens and natural microbiota on surfaces of fresh produce. According to results obtained from MIC and MBC studies, EOCs encapsulated in SDS exert effective antimicrobial activity on pathogens and also possesses high EOC encapsulation capacity. Therefore, SDS was used to encapsulate EOC in this study. From a preliminary experiment (10.1.7.1), spraying and immersion methods did not differ in inactivating pathogens on produce surfaces. In another preliminary assay (11.1.1.1), priming with 70% ethanol prior to application of EOCs -loaded micelles showed significant inhibitory pathogen

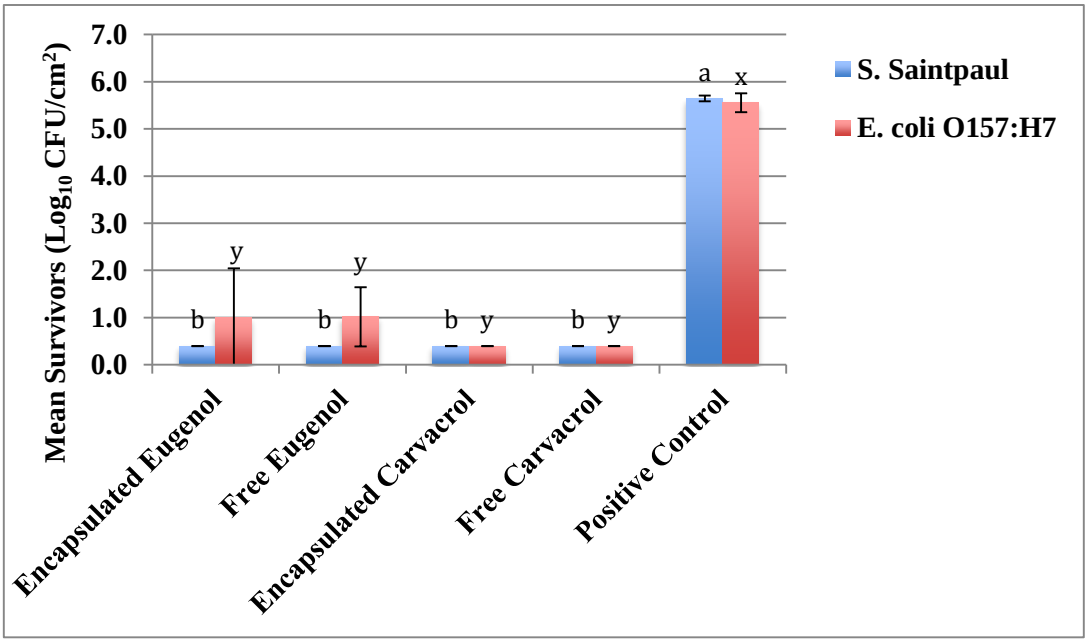

FIGURE 11-3. Mean survivors (Log_{10} CFU/cm^2) of *S.* Saintpaul and *Escherichia coli* O157:H7 on tomato samples treated with encapsulated eugenol, non-encapsulated eugenol, encapsulated carvacrol, and non-encapsulated carvacrol applied via 2 min immersion and stored at 5°C for 0 day. Columns depict means log_{10} reduction from triplicate identical replications (n = 3) while error bars depict standard deviation from the mean. Within pathogen, means not connected with same letter are significantly different ($p<0.05$). The limit of detection is 0.7 log_{10} CFU/cm^2.

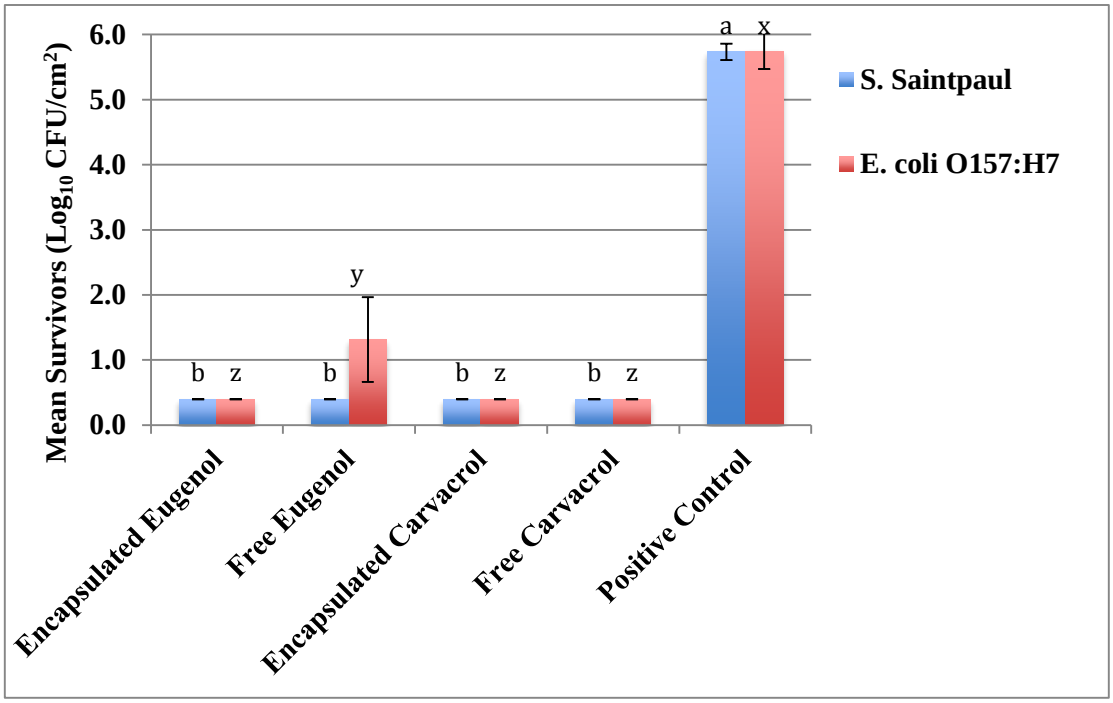

FIGURE 11-4. Mean survivors (Log_{10} CFU/cm^2) of *S.* Saintpaul and *Escherichia coli* O157:H7 on tomato samples treated with encapsulated eugenol, non-encapsulated eugenol, encapsulated carvacrol, and non-encapsulated carvacrol applied via 2 min immersion and stored at 5°C for 2 day. Columns depict means log_{10} reduction from triplicate identical replications (n = 3) while error bars depict standard deviation from the mean. Within pathogen, means not connected with same letter are significantly different ($p<0.05$). The limit of detection is 0.7 log_{10} CFU/cm^2.

inactivation effect inspinach compared to application of EOCs micelles without priming with 70% ethanol. However, treatments with EOCs-loaded micelles without priming yielded undetectable numbers of pathogens on tomatoes, suggesting that priming agent is not necessary for pathogen inactivation on smooth produce surfaces. Thus, priming agent was not used in this study for microbial inactivation for tomato and spinach. The efficacy of encapsulated EOCs versus free EOCs was also studied in the preliminary assay (11.1.1.2). Significant difference in pathogen inactivation effect was observed with encapsulated eugenol versus free eugenol but was not observed with encapsulated carvacrol and free carvacrol. Thus eugenol was used for encapsulation in surfactant micelles for pathogen inhibition assays on tomato and spinach surfaces.

Figure 11-5a represents populations of *S.* Saintpaul on tomato samples (set A) after treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water. Set A of tomato samples was stored at 5°C for the entire 10-day storage period. On day 0, the initial population of *S.* Saintpaul on the positive control sample was 5.8±0.1 $\log_{10}$ CFU/cm^2. After treatments, populations of *S.* Saintpaul ranged from 0.9±0.9 to 5.4±0.1 $\log_{10}$ CFU/cm^2 on day 0. Compared to the initial *S.* Saintpaul population, water did not reduce populations of *S.* Saintpaul ($p \geq 0.05$) on day 0. Populations of *S.* Saintpaul after all other treatments were lower than the positive control on day 0 ($p < 0.05$). The antimicrobial effect of empty micelles was not different from water ($p \geq 0.05$). Encapsulated eugenol, free eugenol, and chlorine were similarly effective ($p \geq 0.05$) in reducing *S.* Saintpaul population and were more effective ($p < 0.05$) than water and empty micelles on day 0. From day 3 to day 10, the populations of *S.*

Saintpaul on tomato samples treated with encapsulated eugenol, free eugenol, empty micelles, and chlorine were significantly lower than the positive controls ($p<0.05$). From days 3 to 10 of storage, encapsulated and free eugenol reduced *S.* Saintpaul levels to below detectable levels. On day 3, chlorine reduced the populations of *S.* Saintpaul to 0.7 ± 0.8 $\log_{10}$ CFU/cm^2 and was less effective than encapsulated and free eugenol ($p<0.05$). On day 5, empty micelles reduced *S.* Saintpaul level to 0.5 ± 0.5 $\log_{10}$ CFU/cm^2 and was less effective than encapsulated and free eugenol ($p<0.05$). Excepting water, all treatments inactivated *S.* Saintpaul populations to below detectable level on day 7 ($p<0.05$). On day 10 of storage, populations of *S.* Saintpaul from encapsulated eugenol, free eugenol, empty micelles, and chlorine did not differ ($p\geq0.05$) and were similar to *S.* Saintpaul populations on day 5 and 7 ($p\geq0.05$). From days 3 to 7 water treatment yielded lower levels of *S.* Saintpaul compared to control ($p<0.05$) but showed the least effective antimicrobial activity compared to other treatments ($p<0.05$). On day 10, water did not reduce *S.* Saintpaul populations ($p\geq0.05$). Compared to the initial population on day 0, *S.* Saintpaul population on the positive control on day 10 significantly decreased to 3.9 ± 0.7 $\log_{10}$ CFU/cm^2.

Populations of *E. coli* O157:H7 on tomato samples (set A) after treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water are shown in Figure 10-8b. On day 0, the initial population of *E. coli* O157:H7 on the positive control was 5.7 ± 0.2 $\log_{10}$ CFU/cm^2. After treatments on day 0, the populations of *E. coli* O157:H7 ranged from 1.5 ± 1.2 to 4.8 ± 0.1 $\log_{10}$ CFU/cm^2. Water did not exert inhibitory

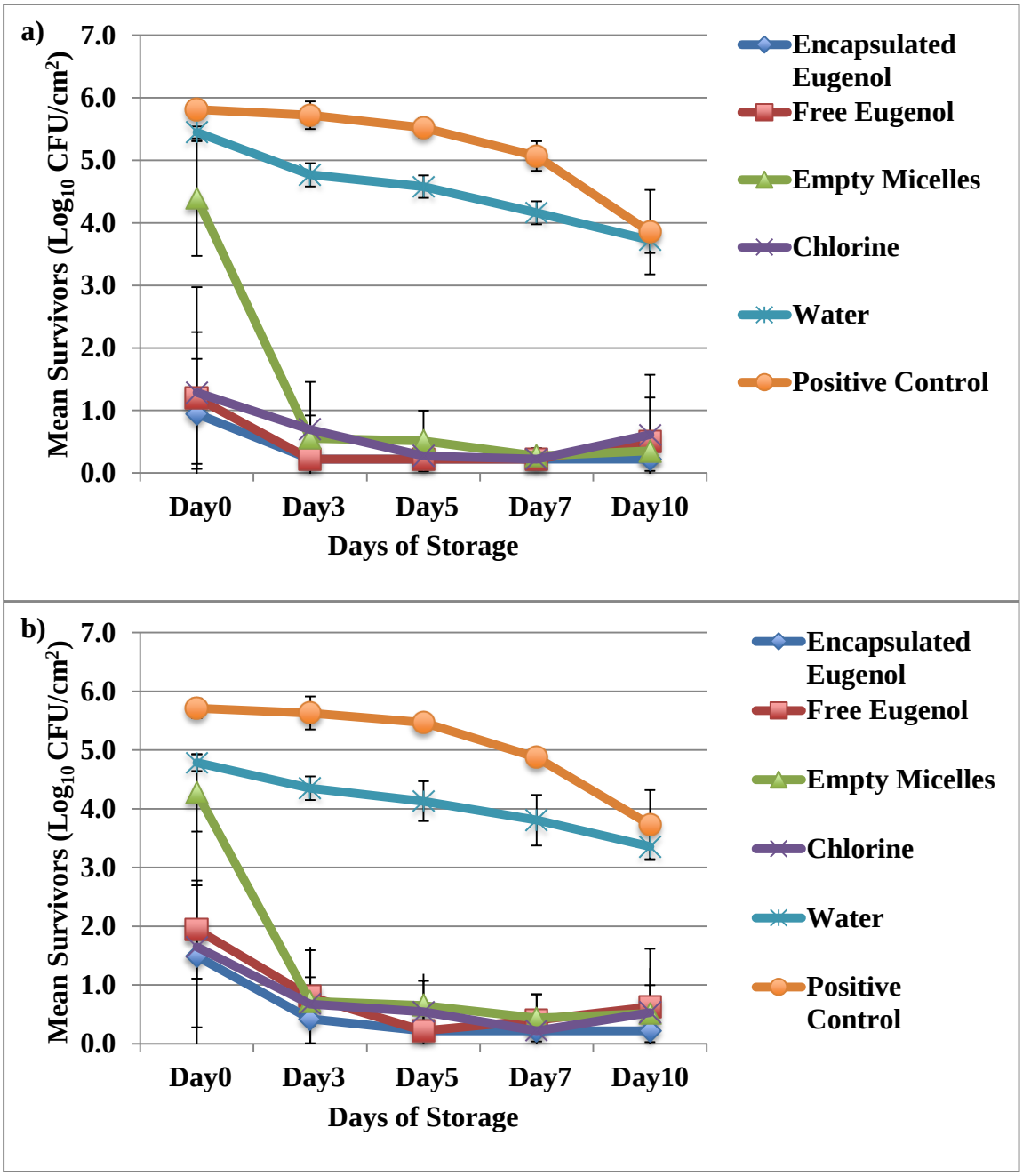

FIGURE 11-5. Survival of a) *Salmonella* Saintpaul and b) *Escherichia coli* O157:H7 numbers on tomato (set A) treated with encapsulated eugenol micelles, free eugenol, empty micelles, chlorine, and water. Samples were via 2 min immersion and and were stored at 5°C for up to 10 days**.** Lines depict means from duplicate triplicate identical replications (n = 6) while error bars depict standard deviation from the mean. Encapsulated eugenol, 1%SDS + 1% eugenol; free eugenol, 1%eugenol; empty micelles ,1%SDS; chlorine, 200 ppm chlorine. The limit of detection is 0.5 $\log_{10}$ CFU/cm^2.

effects against *E. coli* O157:H7 ($p \geq 0.05$) on day 0 and the antimicrobial effect of empty micelles did not differ from water ($p \geq 0.05$).Encapsulated eugenol, free eugenol, and chlorine were similarly effective ($p \geq 0.05$) in reducing *S.* Saintpaul population on day 0 and were more effective than water and empty micelles ($p < 0.05$). From day 3 to 10, populations of *E. coli* O157:H7 after treatment with encapsulated eugenol, free eugenol, empty micelles, and chlorine ranged from undetectable (0.2 ± 0.0 $\log_{10}$ CFU/cm^2) to 0.8 ± 0.8 $\log_{10}$ CFU/cm^2 and did not differ from each other ($p \geq 0.05$). Populations of *E. coli* O157:H7 (3.8 ± 0.4 to 4.4 ± 0.2 $\log_{10}$ CFU/cm^2) after water treatment from day 3 to 7 were lower than positive controls ($p < 0.05$) but were significantly higher than *E. coli* O157:H7 populations from other treatments. On day 10, water did not reduce the level of *E. coli* O157:H7 on tomato samples ($p \geq 0.05$). Compared to the initial population on day 0, *E. coli* O157:H7 level on the positive control on day 10 significantly decreased to 3.7 ± 0.6 $\log_{10}$ CFU/cm^2.

Figure 11-6a depicts populations of *S.* Saintpaul on tomato samples (set B) after treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water. After treatments, the set B tomato samples were initially stored at 5°C and were moved to 15°C on day 5 until day 10. On day 0 of storage, the positive control sample contained *S.* Saintpaul level of 5.8 ± 0.3 $\log_{10}$ CFU/cm^2. After treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water on day 0, the populations of *S.* Saintpaul ranged from 0.5 ± 0.6 to 5.4 ± 0.2 $\log_{10}$ CFU/cm^2. Compared to the positive control, water did not exhibit inhibitory effect on *S.* Saintpaul ($p \geq 0.05$). On day 0, encapsulated eugenol, free eugenol, and chlorine were similarly effective ($p \geq 0.05$) in

reducing *S.* Saintpaul population and were more effective than water and empty micelles ($p<0.05$). From day 3 to 7, treatment with encapsulated eugenol, free eugenol, empty micelles, and chlorine significantly decreased *S.* Saintpaul levels to 0.2±0.0 to 1.9 ±1.0 $\log_{10}$ CFU/cm^2. On day 10, population of *S.* Saintpaul on the positive control was 5.6±0.9 $\log_{10}$ CFU/cm^2 and was not different from the control on day 0 ($p\geq0.05$). *S.* Saintpaul levels from treatments with encapsulated eugenol, free eugenol, and chlorine on day 10 yielded significantly lower ($p<0.05$) populations (0.2±0.0 to 1.6±2.4 $\log_{10}$ CFU/cm^2) than the positive control and their antimicrobial effects did not differ. from each other ($p\geq0.05$). Empty micelles did not reduce the *S.* Saintpaul level (4.6±2.3 $\log_{10}$ CFU/cm^2) on tomato samples on day 10 of storage ($p\geq0.05$). Treatment with water yielded increased population (7.9±0.4 $\log_{10}$ CFU/cm^2) of *S.* Saintpaul compared to the control on day 10 ($p<0.05$).

Figure 11-6b represents populations of *E. coli* O157:H7 on tomato samples (set B) after treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water. On day 0 of storage, populations of *E. coli* O157:H7 after all treatments ranged from 0.6±0.8 to 4.9±0.2 $\log_{10}$ CFU/cm^2 and the population *E. coli* O157:H7 on the control was 5.9±0.3. All treatments on day 0 resulted in *E. coli* O157:H7 populations lower the control ($p<0.05$). The antimicrobial effects of encapsulated and free eugenol on day 0 did not differ ($p\geq0.05$) and were more efficient than chlorine, empty micelles and water ($p<0.05$). On day 0, the antimicrobial effects of empty micelles and water were not different ($p\geq0.05$) and were less effective than chlorine ($p<0.05$). From day 3 to

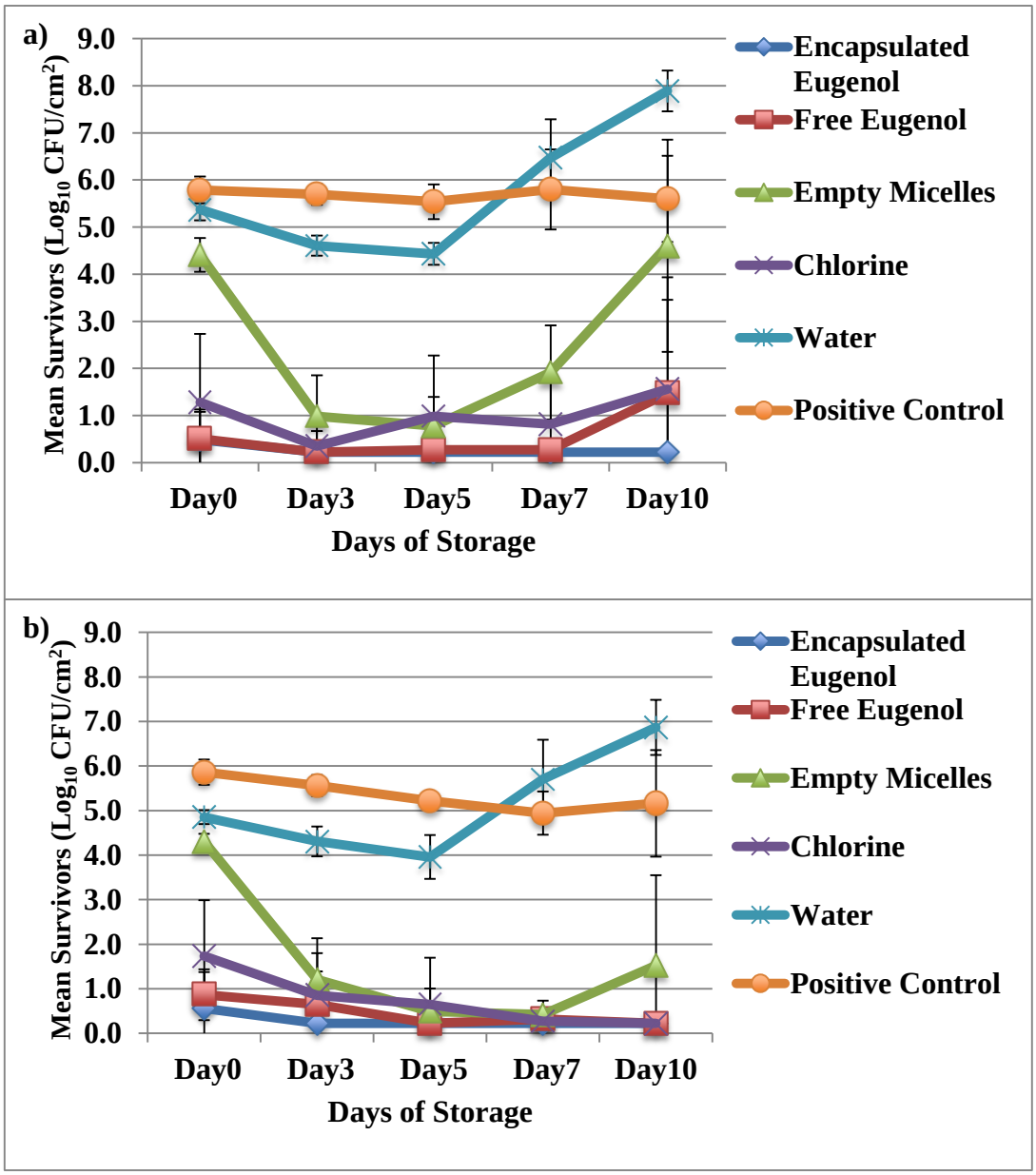

FIGURE 11-6. Survival of a) *Salmonella* Saintpaul and b) *Escherichia coli* O157:H7 numbers on tomato (set B) treated with encapsulated eugenol micelles, free eugenol, empty micelles, chlorine, and water. Samples were treated via 2 min immersion and were initially stored at 5°C. On day 5, samples were transitioned to 15°C. Lines depict means from duplicate triplicate identical replications (n = 6) while error bars depict standard deviation from the mean. Encapsulated eugenol, 1%SDS + 1% eugenol; free eugenol, 1%eugenol; empty micelles ,1%SDS; chlorine, 200 ppm chlorine. The limit of detection is 0.5 $\log_{10}$ CFU/cm^2.

7, populations of *E. coli* O157:H7 after treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine ranged from 0.2±0.0 to 1.2±0.6 $\log_{10}$ CFU/cm^2 and were significantly lower than the control ($p<0.05$). On day 10, the treatments with encapsulated eugenol, free eugenol, and chlorine resulted in undetectable level (0.2±0.0 $\log_{10}$ CFU/cm^2) of *E. coli* O157:H7. Empty micelles reduced the level of *E. coli* O157:H7 to 1.5±2.0 $\log_{10}$ CFU/cm^2 on day10 and was less effective than encapsulated eugenol, free eugenol, and chlorine. Compared to positive controls, water reduced levels of *E. coli* O157:H7 on day 3 and 5 ($p<0.05$). However, on day 7 and 10, water treatment increased populations of *E. coli* O157:H7 to 5.7±0.9 and 6.9±0.6 $\log_{10}$ CFU/cm^2 respectively ($p<0.05$). The level of *E. coli* O157:H7 on the positive controls did not change over 10 days of storage ($p<0.05$).

Populations of *S.* Saintpaul and *E. coli* O157:H7 from set A and B of tomato samples are shown in Table 11-1. On day 5, set B of tomato samples was transitioned from 5°C to 15°C to simulate temperature abuse condition while set A samples remained at 5°C. On day 7, levels of both pathogens on set A samples did not differ from levels on set B samples ($p\geq0.05$). Treatment with empty micelles resulted in higher populations of *S.* Saintpaul on set B tomato compared to set A tomato on day 7 ($p<0.05$). Water treatment yielded higher populations of both pathogens on set B samples compared to populations on set A samples on day 7 and 10 ($p<0.05$). On day 10, higher levels of both pathogens on set B of tomato samples were observed ($p<0.05$). Excepting encapsulated eugenol, higher levels of *S.* Saintpaul populations on set B of tomato samples were

TABLE 11-1. Survival of *Salmonella* Saintpaul and *Escherichia coli* O157:H7 (log_{10} CFU/cm^2) on set A and B of tomato samples[a] after treatment with encapsulated eugenol, empty micelles, chlorine, and water.[b]

Treatment		*Salmonella* Saintpaul		*Escherichia coli* O157:H7	
	Days of Storage	Set A	Set B	Set A	Set B
Encapsulated Eugenol	0	0.9 ± 0.9^{lmnop}	0.5 ± 0.6^{nop}	1.5 ± 1.2^{jk}	0.6 ± 0.8^{lm}
Free Eugenol	0	1.2 ± 1.1^{klmno}	0.5 ± 0.6^{nop}	1.9 ± 0.8^{j}	0.9 ± 0.6^{klm}
Empty Micelles	0	4.4 ± 0.9^{ghij}	4.4 ± 0.4^{ghij}	4.3 ± 0.7^{fgh}	4.3 ± 0.2^{fgh}
Chlorine	0	1.3 ± 1.7^{klmn}	1.3 ± 1.4^{klmn}	1.7 ± 2.0^{j}	1.7 ± 1.3^{j}
Water	0	5.4 ± 0.1^{cde}	5.4 ± 0.2^{cdef}	4.8 ± 0.1^{efg}	4.9 ± 0.2^{defg}
Positive Control	0	5.8 ± 0.1^{bc}	5.8 ± 0.3^{bc}	5.7 ± 0.2^{b}	5.9 ± 0.3^{b}
Encapsulated Eugenol	3	0.2 ± 0.0^{p}	0.2 ± 0.0^{p}	0.4 ± 0.5^{m}	0.2 ± 0.0^{m}
Free Eugenol	3	0.2 ± 0.0^{p}	0.2 ± 0.0^{p}	0.8 ± 0.8^{klm}	0.7 ± 0.7^{lm}
Empty Micelles	3	0.6 ± 0.4^{nop}	1.0 ± 0.9^{lmnop}	0.7 ± 0.4^{lm}	1.2 ± 0.6^{jkl}
Chlorine	3	0.7 ± 0.8^{mnop}	0.4 ± 0.3^{op}	0.7 ± 1.0^{lm}	0.9 ± 1.3^{klm}
Water	3	4.8 ± 0.2^{defgh}	4.6 ± 0.2^{efghi}	4.4 ± 0.2^{fgh}	4.3 ± 0.3^{fgh}
Positive Control	3	5.7 ± 0.2^{bc}	5.7 ± 0.2^{bc}	5.6 ± 0.3^{bc}	5.6 ± 0.2^{bcd}
Encapsulated Eugenol	5	0.2 ± 0.0^{p}	0.2 ± 0.0^{p}	0.2 ± 0.0^{m}	0.2 ± 0.0^{m}
Free Eugenol	5	0.2 ± 0.0^{p}	0.3 ± 0.1^{p}	0.2 ± 0.0^{m}	0.2 ± 0.0^{m}
Empty Micelles	5	0.5 ± 0.5^{nop}	0.8 ± 0.6^{lmnop}	0.7 ± 0.4^{lm}	0.5 ± 0.5^{lm}
Chlorine	5	0.3 ± 0.1^{p}	1.0 ± 1.3^{lmnop}	0.5 ± 0.1^{lm}	0.6 ± 1.0^{lm}
Water	5	4.6 ± 0.2^{fghij}	4.4 ± 0.2^{ghij}	4.1 ± 0.3^{gh}	4.0 ± 0.5^{hi}
Positive Control	5	5.5 ± 0.2^{cd}	5.5 ± 0.4^{cd}	5.5 ± 0.1^{bcde}	5.2 ± 0.2^{bcde}
Encapsulated Eugenol	7	0.2 ± 0.0^{p}	0.2 ± 0.0^{p}	0.2 ± 0.0^{m}	0.2 ± 0.0^{m}
Free Eugenol	7	0.2 ± 0.0^{p}	0.3 ± 0.1^{p}	0.4 ± 0.4^{m}	0.3 ± 0.2^{m}
Empty Micelles	7	0.3 ± 0.1^{p}	1.9 ± 1.0^{k}	0.4 ± 0.4^{m}	0.4 ± 0.3^{m}
Chlorine	7	0.2 ± 0.0^{p}	0.8 ± 1.0^{lmnop}	0.2 ± 0.0^{m}	0.3 ± 0.1^{m}
Water	7	4.2 ± 0.2^{hij}	6.5 ± 0.8^{b}	3.8 ± 0.4^{hi}	5.6 ± 0.9^{b}
Positive Control	7	5.1 ± 0.2^{cdefg}	5.8 ± 0.9^{bc}	4.9 ± 0.1^{cdefg}	4.9 ± 0.5^{cdef}
Encapsulated Eugenol	10	0.2 ± 0.0^{p}	0.2 ± 0.0^{p}	0.2 ± 0.0^{m}	0.2 ± 0.0^{m}
Free Eugenol	10	0.5 ± 0.7^{nop}	1.5 ± 2.0^{klm}	0.6 ± 1.0^{lm}	0.2 ± 0.0^{m}
Empty Micelles	10	0.4 ± 0.3^{op}	4.6 ± 2.3^{efghi}	0.5 ± 0.5^{lm}	1.5 ± 2.0^{jk}
Chlorine	10	0.6 ± 1.0^{nop}	1.6 ± 2.4^{kl}	0.5 ± 0.8^{lm}	0.2 ± 0.0^{m}
Water	10	3.7 ± 0.2^{j}	7.9 ± 0.4^{a}	3.4 ± 0.2^{i}	6.9 ± 0.6^{a}
Positive Control	10	3.9 ± 0.7^{ij}	5.6 ± 0.9^{cd}	3.7 ± 0.6^{hi}	5.2 ± 1.2^{bcde}

[a]After treatments, set A of tomato samples was stored at 5°C for the entire 10-day storage period. Set B of tomato samples was initially stored at 5°C after treatments and were transitioned to 15°C on day 5 until day 10.

[b]Data represent means ± standard deviations from duplicate triplicate identical replications (n = 6). Across both sample sets, means within the same pathogen grouping not connected by same letter are significantly different ($p<0.05$).

observed with all treatments on day 10. Higher level of *E. coli* O157:H7 on set B of tomato samples was observed with empty micelles treatment on day 10 ($p<0.05$).

Populations of aerobic bacteria, Enterobacteriaceae, and yeasts and molds on tomato samples (set A) after treatment with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water are shown in Figure 11-7. After treatments, samples were stored at 5°C for up to 10 days. On day 0 of storage, initial populations of aerobic bacteria, Enterobacteriaceae, and yeasts and molds were 1.3±1.0, 0.8±1.2, and 2.9±0.4 $\log_{10}$ CFU/cm^2 respectively (Figure 11-7a to 11-7c). On day 0 of storage, treatments with encapsulated eugenol, free eugenol, and chlorine reduced populations of aerobic bacteria to 0.3±0.1 to 0.5±0.4 $\log_{10}$ CFU/cm^2 ($p<0.05$) (Figure 11-7a). Water did not reduce population of aerobic bacteria on day 0 ($p\geq0.05$). From day 3 to 10, populations of aerobic bacteria after treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water ranged from 0.3±0.1 to 2.3±2.5 $\log_{10}$ CFU/cm^2. From day 5 today 7, all treatments did not reduce levels of aerobic bacteria during storage ($p\geq0.05$). On day 10, only encapsulated eugenol decreased the level of aerobic bacteria ($p<0.05$). Levels of aerobic bacteria on untreated tomato samples did not change over 10 days of storage ($p\geq0.05$). For Enterobacteriaceae, the populations after all treatments did not differ from the controls throughout 10 days of storage ($p\geq0.05$) (Figure 11-7b). From day 0 to day 10 of storage, treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water resulted in populations of yeasts and molds ranging from 0.5±0.3 to 3.6±0.9 $\log_{10}$ CFU/cm^2 (Figure 10-10c). On day 0, excepting empty micelles,

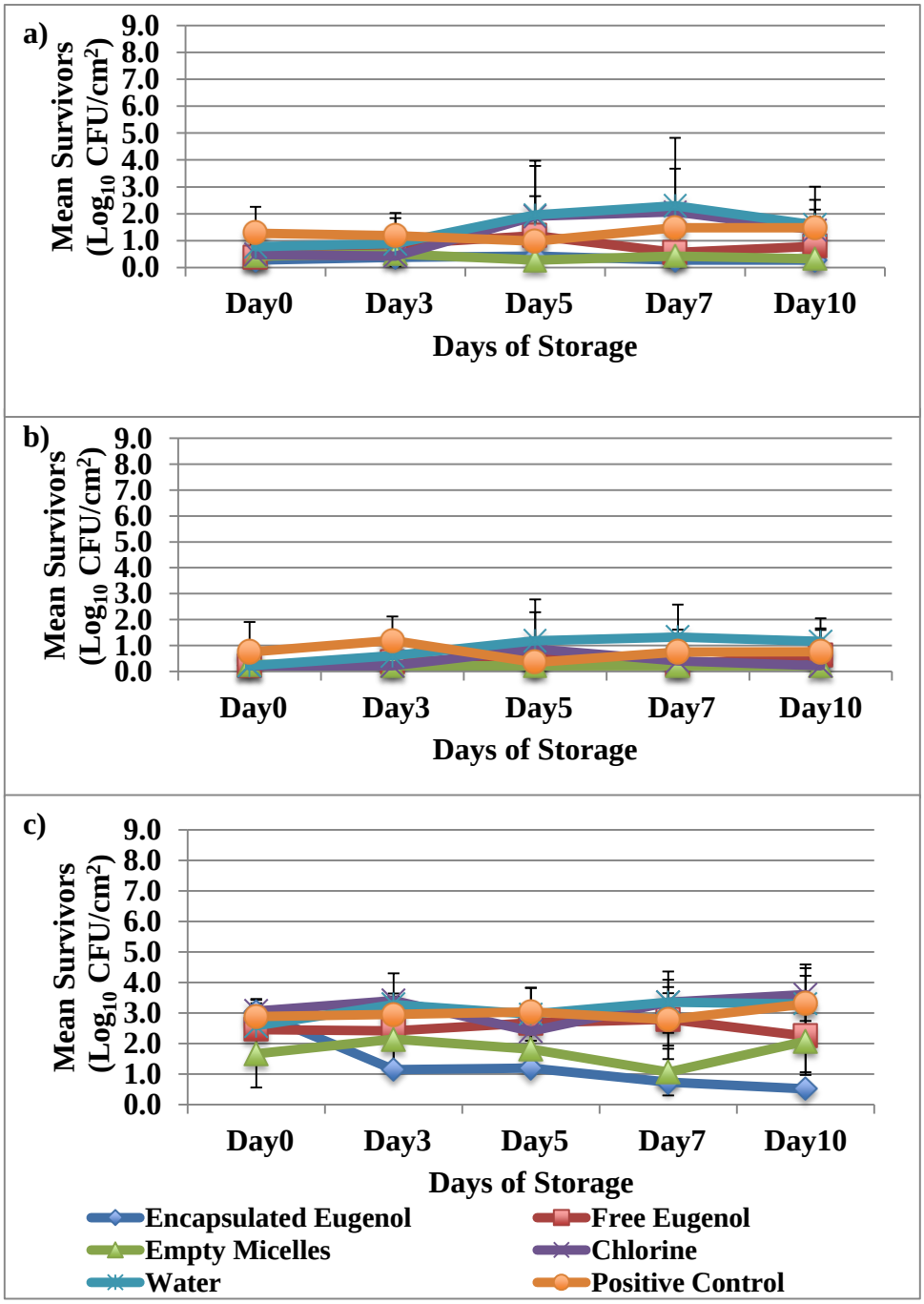

FIGURE 11-7. Survival of a) aerobic bacteria, b) Enterobacteriaceae, c) yeasts and molds on tomato (set A) treated with encapsulated eugenol micelles, free eugenol, empty micelles, chlorine, and water. Samples were treated via 2 min immersion and were stored at 5°C for up to 10 days. Lines depict means from duplicate triplicate identical replications (n = 6) while error bars depict standard deviation from the mean. The limit of detection is 0.5 $\log_{10}$ CFU/cm^2.

all treatments did not reduce the levels of yeasts and molds on tomato samples ($p \geq 0.05$). and empty On day 3, only encapsulated eugenol decreased the population of yeasts and molds ($p < 0.05$). After day 3 until day 10 of storage, only encapsulated eugenol micelles significantly reduced the levels of yeasts and molds ($p < 0.05$) tomato samples ($p \geq 0.05$). On day 3, only encapsulated eugenol decreased the population of yeasts and molds ($p < 0.05$). After day 3 until day 10 of storage, only encapsulated eugenol and empty micelles significantly reduced the levels of yeasts and molds ($p < 0.05$).

Figure 11-8 depicts populations of aerobic bacteria, Enterobacteriaceae, and yeasts and molds on on tomato samples (set B) after treatment with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water. After treatments, samples were initially stored at 5°C and were moved to 15°C on day 5 until day 10 of storage. On day 0, the initial populations populations of aerobic bacteria, Enterobacteriaceae, and yeasts and molds on tomato samples were 2.0±1.5, 1.6±1.7, and 2.9±0.4 $\log_{10}$ CFU/cm^2 On day 0, after treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water, the levels of aerobic bacteria on tomato samples ranged from 0.3±0.1 to 1.3±1.6 $\log_{10}$ CFU/cm^2. Encapsulated eugenol, chlorine, and empty micelles were similarly effective ($p \geq 0.05$) in reducing aerobic bacteria levels on day 0 while water and free eugenol did not reduce aerobic bacteria levels ($p \geq 0.05$) (Figure 10-11a). Populations after treatments from day 3 to day 10 ranged from 0.4±0.3 to 6.3±1.7 $\log_{10}$ CFU/cm^2. On day 3 of storage, populations of aerobic bacteria from all treatments did not differ from the control ($p \geq 0.05$). On day 5, only encapsulated eugenol reduced the population of aerobic bacteria on tomato samples ($p < 0.05$). Excepting water, all

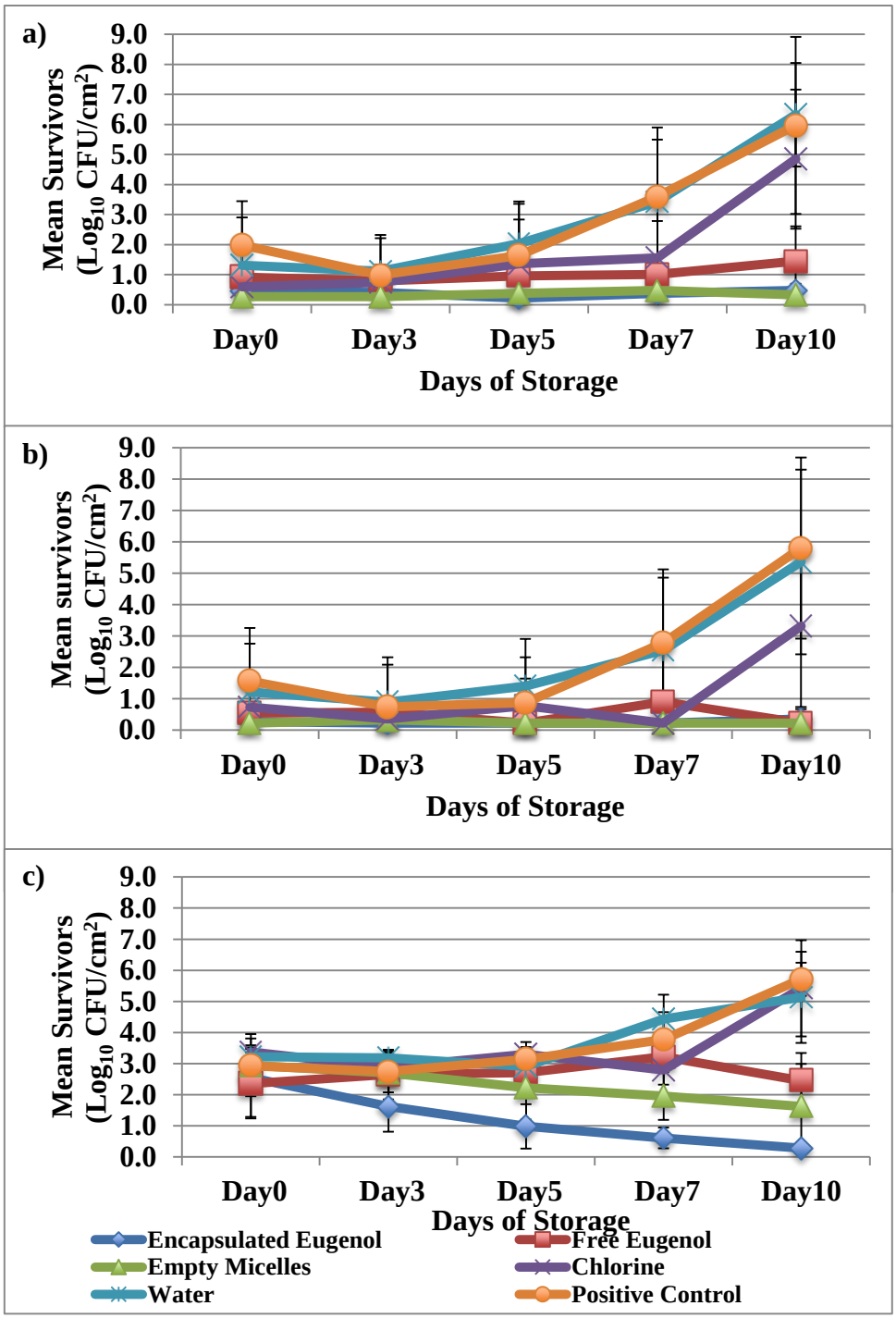

FIGURE 11-8. Survival of a) aerobic bacteria, b) Enterobacteriaceae, and c) yeasts and molds on tomato (set B) treated with encapsulated eugenol micelles, free eugenol, empty micelles, chlorine, and water. Samples were treated via 2 min immersion and were initially stored at 5°C and transitioned to 15°C on day 5. Lines depict means from duplicate triplicate identical replications (n = 6) while error bars depict standard deviation from the mean. The limit of detection is 0.5 $\log_{10}$ CFU/cm^2.

treatments reduced levels of aerobic bacteria on day 7 ($p<0.05$) and their antimicrobial effect did not differ ($p\geq0.05$). On day 10, encapsulated eugenol, free eugenol, and empty micelles were similarly effective in reducing aerobic bacteria populations on tomato samples ($p<0.05$); however, water and chlorine did not show inhibitory effect on aerobic bacteria populations ($p\geq0.05$). The levels of aerobic bacteria on positive control did not change from day 0 to day 5 ($p\geq0.05$) and increased after day 5 of storage ($p<0.05$). On day 0, empty micelles and encapsulated eugenol reduced levels of Enterobacteriaceae to 0.2±0.0 and 0.3±0.1 $\log_{10}$ CFU/cm^2 respectively. Other treatments did not reduce the numbers of Enterobacteriaceae on tomato samples on day 0. On day 3 and 5, numbers of Enterobacteriaceae from all treatments did not differ from the untreated sample ($p\geq0.05$). Excepting water, all treatments reduced numbers of Enterobacteriaceae on tomatoes on day 7 and 10 ($p<0.05$). Levels of Enterobacteriaceae on untreated samples did not change from day 0 to day 5 ($p\geq0.05$) but significantly increased to 2.8±2.1 and 5.8±2.9 $\log_{10}$ CFU/cm^2 on day 7 and 10 respectively ($p<0.05$). Numbers of yeasts and molds after treatments on day 1 ranged from 2.4±1.1 to 3.2±0.3 $\log_{10}$ CFU/cm^2 and did not differ from the positive control ($p\geq0.05$). Only encapsulated eugenol reduced populations of yeasts and molds on day 3($p<0.05$). On day 5, encapsulated eugenol and empty micelles decreased populations of yeasts ad molds ($p<0.05$). Populations of yeasts and molds on untreated samples did not significantly change ($p\geq0.05$) from day 0 to day 7 of storage but increased on day 10 ($p<0.05$). Encapsulated eugenol, empty micelles and chlorine significantly reduced numbers of yeasts and molds on day 7. Populations of yeasts and molds were reduced by encapsulated eugenol, free eugenol and empty micelles ($p<0.05$)

but not by water and chlorine ($p \geq 0.05$) on day 10.

Table 11-2 shows populations of aerobic bacteria, Enterobacteriaceae and yeasts and molds on set A and B of tomato samples. Compared to set A, storing set B of tomato samples at 15°C yielded higher populations of aerobic bacteria, Enterobacteriaceae, and yeasts and molds on day 7 and 10 ($p<0.05$). Excepting higher populations of Enterobacteriaceae and yeasts and molds on set B of tomato samples, numbers of all native microbiota did not differ between set A and set B on day 7 ($p \geq 0.05$). On day 10, water and chlorine treatment resulted in higher populations of aerobic bacteria, Enterobacteriaceae, and yeasts and molds of set B samples compared to set A samples ($p<0.05$). Differences of aerobic bacteria, Enterobacteriaceae, and yeasts and molds between two sample sets were not observed with other treatments on day 10 ($p \geq 0.05$).

Overall, for pathogen inactivation on tomato surfaces, all treatments followed the trend from greatest to least antimicrobial effect of encapsulated eugenol = free eugenol = chlorine ≥ empty micelles > water. Encapsulated eugenol, free eugenol, chlorine and empty micelles showed efficient residual effects in reducing pathogen populations to below or just over detectable levels after day 0 of storage when tomato samples were stored at 5°C for the entire 10-day storage period. However, treatment with empty micelles showed less efficient antimicrobial effect when tomato samples were shifted to 15°C. Water also increased populations of pathogens when tomato samples were stored at 15°C. For native microbiota, in general, all treatments excepting water were similarly effective in reducing aerobic bacteria and Enterobacteriaceae populations during storage at 5°C. When tomato samples were shifted to 15°C, chlorine showed less efficient

TABLE 11-2. Survival of aerobic bacteria, Enterobacteriaceae, and yeasts and molds (log_{10} CFU/cm^2) on set A and B of tomato samples[a] after treatment with encapsulated eugenol, empty micelles, chlorine, and water.[b]

Treatment	Days of Storage	Aerobic Bacteria		Enterobacteriaceae		Yeasts and Molds	
		Set A	Set B	Set A	Set B	Set A	Set B
Encapsulated Eugenol	0	0.3 ± 0.1^{jk}	0.5 ± 0.3^{ijk}	0.2 ± 0.0^{e}	0.3 ± 0.1^{e}	3.0 ± 0.4^{defghijkl}	2.5 ± 1.3^{fghijklmnopq}
Free Eugenol	0	0.4 ± 0.2^{ijk}	0.9 ± 0.9^{fghijk}	0.2 ± 0.0^{e}	0.5 ± 0.8^{de}	2.5 ± 1.0^{fghijklmnopq}	2.4 ± 1.1^{ijklmnopq}
Empty Micelles	0	0.4 ± 0.2^{ijk}	0.3 ± 0.1^{jk}	0.3 ± 0.1^{e}	0.2 ± 0.0^{e}	1.7 ± 1.1^{pqrstuv}	2.9 ± 1.0^{defghijklm}
200 ppm Chlorine	0	0.5 ± 0.4^{ijk}	0.6 ± 0.4^{hijk}	0.2 ± 0.0^{e}	0.7 ± 0.9^{de}	3.1 ± 0.4^{defghijkl}	3.4 ± 0.2^{defg}
Water	0	0.8 ± 0.3^{fghijk}	1.3 ± 1.6^{efghijk}	0.2 ± 0.0^{e}	1.2 ± 1.5^{de}	2.6 ± 0.5^{fghijklmnop}	3.2 ± 0.3^{defghij}
Positive Control	0	1.3 ± 1.0^{efghijk}	2.0 ± 1.5^{efg}	0.8 ± 1.2^{de}	1.6 ± 1.7^{cd}	2.9 ± 0.4^{defghijklmn}	2.9 ± 0.4^{defghijklm}
Encapsulated Eugenol	3	0.4 ± 0.2^{ijk}	0.4 ± 0.3^{ijk}	0.2 ± 0.0^{e}	0.2 ± 0.0^{e}	1.1 ± 0.9^{stuvw}	1.6 ± 0.8^{qrstuv}
Free Eugenol	3	0.9 ± 0.6^{fghijk}	0.8 ± 0.3^{fghijk}	0.4 ± 0.3^{e}	0.6 ± 0.3^{de}	2.4 ± 1.2^{hijklmnopq}	2.6 ± 0.8^{efghijklmno}
Empty Micelles	3	0.5 ± 0.3^{ijk}	0.3 ± 0.1^{jk}	0.2 ± 0.0^{e}	0.2 ± 0.0^{e}	2.1 ± 0.9^{lmnopqr}	2.7 ± 0.3^{efghijklmno}
Chlorine	3	0.4 ± 0.4^{ijk}	0.7 ± 0.6^{ghijk}	0.2 ± 0.0^{e}	0.4 ± 0.4^{e}	3.4 ± 0.9^{def}	2.9 ± 0.5^{defghijklmn}
Water	3	0.9 ± 0.9^{fghijk}	1.1 ± 1.2^{efghijk}	0.6 ± 0.8^{de}	1.2 ± 1.4^{cde}	3.3 ± 0.3^{defghi}	3.2 ± 0.2^{defghij}

TABLE 11-2. Continued.

Treatment	Days of Storage	Aerobic Bacteria		Enterobacteriaceae		Yeasts and Molds	
		Set A	Set B	Set A	Set B	Set A	Set B
Positive Control	3	1.2±0.8^{efghijk}	1.0±1.2^{efghijk}	1.2±0.9^{e}	1.0±1.4^{de}	3.0±0.4^{defghijklm}	2.7±0.7^{efghijklmno}
Encapsulated Eugenol	5	0.4±0.2^{ijk}	0.2±0.0^{k}	0.2±0.0^{e}	0.2±0.0^{e}	1.2±1.4^{rstuvw}	1.0±0.7^{uvw}
Free Eugenol	5	1.2±1.5^{efghijk}	1.0±1.0^{fghijk}	0.5±0.8^{de}	0.2±0.0^{e}	2.7±1.1^{efghijklmno}	2.7±0.7^{efghijklmno}
Empty Micelles	5	0.3±0.1^{jk}	0.4±0.3^{ijk}	0.2±0.0^{e}	0.2±0.0^{e}	1.8±0.7^{opqrstu}	2.2±1.0^{klmnopq}
Chlorine	5	1.9±2.1^{efgh}	1.4±2.0^{efghijk}	0.9±1.4^{de}	0.8±0.9^{de}	2.4±0.8^{hijklmnopq}	3.3±0.4^{defghi}
Water	5	2.0±1.8	2.0±1.4^{efg}	1.2±1.6^{de}	1.4±1.5^{cde}	3.0±0.9^{defghijklm}	2.9±0.5^{defghijklm}
Positive Control	5	1.0±0.6^{efghijk}	1.6±1.2^{efghi}	0.4±0.3^{e}	0.9±1.5^{de}	3.0±0.3^{defghijkl}	3.1±0.4^{defghijk}
Encapsulated Eugenol	7	0.3±0.1^{jk}	0.4±0.2^{ijk}	0.3±0.1^{e}	0.2±0.0^{e}	0.7±0.8^{vw}	0.6±0.3^{w}
Free Eugenol	7	0.6±0.5^{ijk}	1.0±1.8^{efghijk}	0.3±0.1^{e}	0.9±1.7^{de}	2.8±1.3^{efghijklmn}	3.2±0.4^{defghi}
Empty Micelles	7	0.4±0.2^{ijk}	0.5±0.3^{ijk}	0.2±0.0^{e}	0.2±0.0^{e}	1.1±0.8^{tuvw}	2.0±0.8^{nopqrst}
Chlorine	7	2.1±1.6^{ef}	1.6±1.8^{efghij}	0.4±0.4^{de}	0.2±0.0^{e}	3.4±0.5^{defgh}	2.8±0.5^{efghijklmn}
Water	7	2.3±2.5^{de}	3.4±2.1^{d}	1.3±1.3^{de}	2.5±2.6^{bc}	3.4±1.0^{defgh}	4.4±0.8^{bc}

TABLE 11-2. Continued.

Treatment	Days of Storage	Aerobic Bacteria		Enterobacteriaceae		Yeasts and Molds	
		Set A	Set B	Set A	Set B	Set A	Set B
Positive Control	7	1.5±0.8^{efghijk}	3.6±2.3^{cd}	0.8±0.9^{de}	2.8±2.1^{b}	2.8±0.9^{efghijklmn}	3.8±0.9^{cd}
Encapsulated Eugenol	10	0.3±0.1^{ijk}	0.5±0.2^{ijk}	0.3±0.1^{e}	0.4±0.3^{e}	0.5±0.3^{w}	0.3±0.1^{w}
Free Eugenol	10	0.8±1.1^{fghujk}	1.4±1.2^{efghijk}	0.6±1.0^{de}	0.2±0.0^{e}	2.2±1.2^{jklmnopq}	2.5±0.9^{ghijklmnopq}
Empty Micelles	10	0.3±0.2^{ijk}	0.3±0.2^{ijk}	0.2±0.0^{e}	0.2±0.0^{e}	2.1±1.1^{mnopqrs}	1.6±1.4^{qrstuv}
Chlorine	10	1.4±1.6^{efghijk}	4.8±2.3^{bc}	0.2±0.0^{e}	3.3±2.6^{b}	3.6±0.9^{cde}	5.4±1.5^{a}
Water	10	1.6±0.9^{efghij}	6.3±1.7^{a}	1.2±0.9^{de}	5.4±2.9^{a}	3.3±1.3^{defghi}	5.1±1.5^{ab}
Positive Control	10	1.5±0.7^{efghijk}	6.0±2.9^{ab}	0.8±0.8^{de}	5.8±2.9^{a}	3.3±0.9^{defghi}	5.7±0.5^{a}

[a]After treatments, set A of tomato samples was stored at 5°C for the entire 10-day storage period. Set B of tomato samples was initially stored at 5C after treatments and were transitioned to 15°C on day 5 until day 10.

[b]Data represent means ± standard deviations from duplicate triplicate identical replications (n = 6). Across both sample sets, means within the same pathogen grouping not connected by same letter are significantly different ($p<0.05$).

antibacterial activities compared to encapsulated eugenol, free eugenol, and empty micelles. For yeasts and molds on tomato surfaces, overall, treatments followed the trend from greatest to least antifungal effect of encapsulated eugenol > empty micelles ≥ free eugenol > chlorine = water.

Reported mechanisms of action of EOCs against microorganisms include cytoplasmic membrane disruption, destabilization of proton motive force, disturbed electron flow, active transport, coagulation of the cell contents, and suppression of gene expression of various pathogenesis elements *(137, 146, 157, 212, 235, 236)*. The functions of surfactant micelles in delivering an antimicrobial to pathogens can include: 1) improved dispersion of EOC in aqueous phase; 2) transport of EOCs to microbial membranes, and; 3) disruption of microbial membranes to enhance uptake of EOC *(7, 79, 110, 128, 159, 224)*. However, in this study, the antibacterial effect of encapsulated eugenol did not differ from free eugenol. Eugenol is a phenolic compound that is slightly soluble in water (0.64 g/l) *(23)*, so it could be partially dissolved and dispersed in wash water. Mattson *et al. (156)* reported inactivation of *Salmonella* spp. on tomatoes by ~6.0 $\log_{10}$ CFU/ml by 0.75% eugenol applied via immersion for 1 min, suggesting efficient antimicrobial effect of free eugenol in wash water. In the current study, 1% SDS was used as a surfactant to encapsulate 1% eugenol for inactivating pathogens and native microbiota on surfaces of tomatoes and the antimicrobial effect of empty micelles (1% SDS) was also studied. It has been reported that SDS can denature membrane-located proteins and damage the membranes of microbial cells, resulting in leakage of cytoplasmic contents and depolarization of the membrane *(252, 259, 260)*. The surface

of tomato is smooth and hydrophobic *(262)*; thus, water and chlorine do not wet surfaces of tomato efficiently. SDS is a surfactant that can lower surface tension of aqueous solution *(177)*. In pure water, the surface tension of 0.03M (~1%) SDS was reported to to be 36.1 dynes/cm *(177)*. With low surface tension, encapsulated eugenol and empty micelles can wet and cover surfaces of tomato effectively. Chlorine is a strong oxidizing agent and has been widely used for fresh produce decontamination in the range of 50 to 200 ppm in the industry *(59, 90)*. It has been reported that the presence of organic substances in chlorine solution results in formation of toxic substances (e.g. chloramine, trihalomethane) as well as reduced antimicrobial activity *(59)*. Hypochlorous acid (HOCl) is the form of free available chlorine that exerts the highest bactericidal activity against a wide range of microorganisms *(90)*. To maintain available HOCl, the pH of the solution must be in the range of 6.0 to 7.5 *(59)*. In this study, the pH of chlorine solution was adjusted to 7.0. Also distilled water was used to prepare the chlorine solution so the presence of organic matters was very low. Thus, chlorine showed efficient antibacterial effect in reducing pathogens and microbiota on fresh produce in the study. However, compared to encapsulated and free eugenol, chlorine was less effective in reducing yeasts and molds at temperature abuse condition. The effectiveness of chlorine in reducing pathogens on smooth-surface produce commodities has been reported. Mattson et al. *(156)* reported that immersion for 3 min in 100 ppm of chlorine reduced *Salmonella* spp. on tomatoes by 4.0 $\log_{10}$ CFU/ml. Iturriaga and Escartin *(130)* reported 5.0 and 4.5 $\log_{10}$ reduction of *S.* Montevideo after treatment with 1000 and 200 ppm of chlorine respectively. Yuk et al. reported 2 $\log_{10}$ reduction of a five cocktailed strains of

Salmonella serovars *(258)* on surfaces of bell pepper using 200 ppm chlorine in a simulated flume tank for 60 and 120 s. The current study showed that storing water-washed tomatoes at temperature abuse condition elevated numbers of pathogens and microbiota. This could have been due to elevated water activity and elevated temperature that supported microbial growth effectively. According to previous studies water was ineffective in reducing microbial populations on surfaces of fresh produce *(130, 156, 258).*

11.2.3.2 Spinach

Figure 11-9a shows populations of *S.* Saintpaul on spinach samples (set A) after treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water. The set A of spinach samples was stored at 5°C for the entire 10-day storage period.

The population on the positive control on day 0 of storage was 6.0±0.1 $\log_{10}$ CFU/cm^2. On day 0, populations of *S.* Saintpaul after treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water were 1.8±1.2 to 5.6±0.3 $\log_{10}$ CFU/cm^2. Free eugenol was similarly effective as chlorine and was more effective ($p<0.05$) than encapsulated eugenol in reducing populations of *S.* Saintpaul while empty micelles and water did not reduce populations of *S.* Saintpaul ($p\geq0.05$) on day 0. From day 3 to 10, treatments resulted in *S.* Saintpaul ranging from 0.2±0.0 to 5.2±0.2 $\log_{10}$ CFU/cm^2. On day 3, populations of *S.* Saintpaul after all treatments were lower than the control. Encapsulated eugenol, free eugenol and chlorine were similarly effective ($p\geq0.05$) in reducing *S.* Saintpaul populations and were more effective than empty micelles and water ($p<0.05$) on day 3. Encapsulated eugenol and free eugenol yielded undetectable

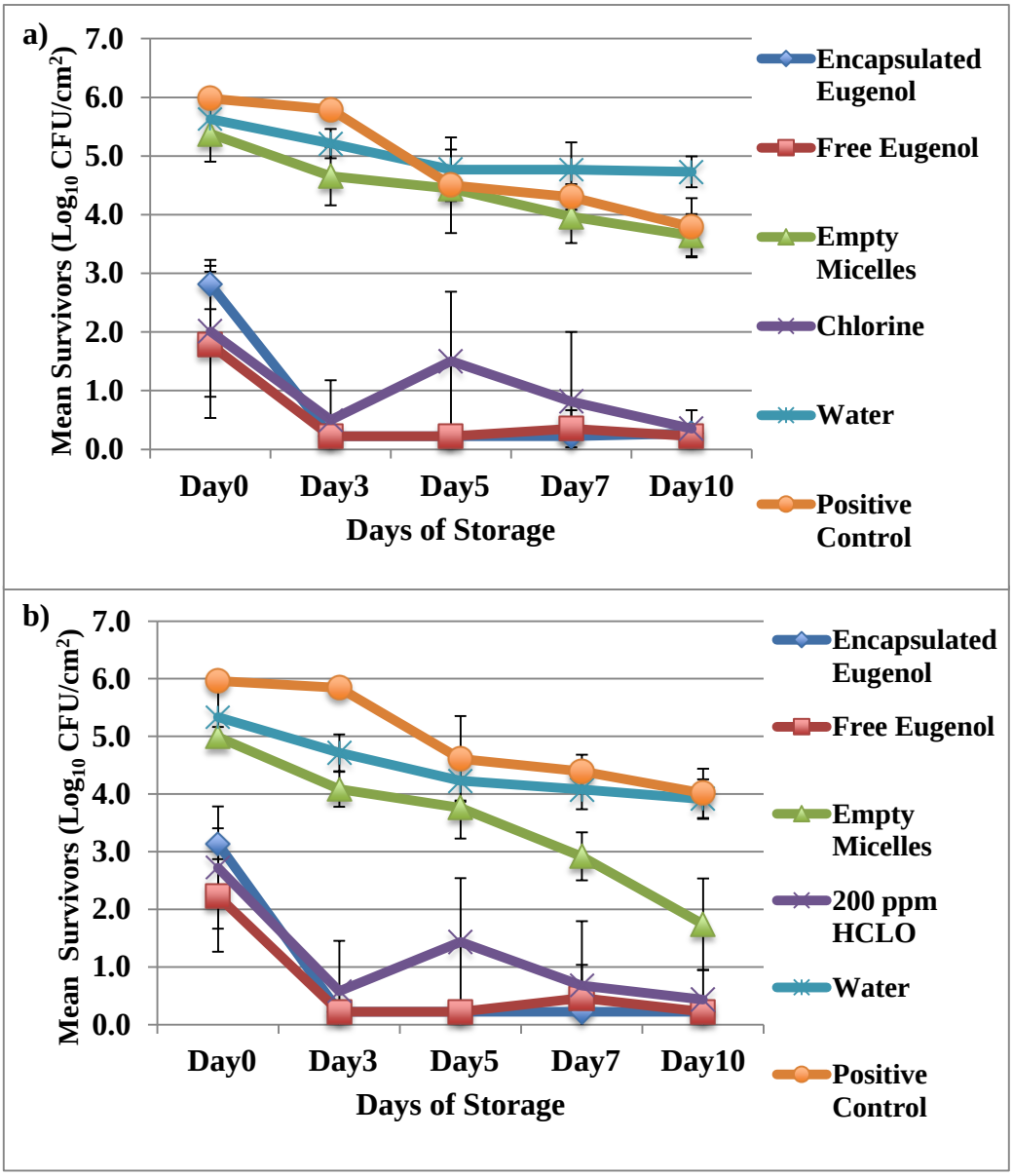

FIGURE 11-9. Survival of a) *Salmonella* Saintpaul and b) *Escherichia coli* O157:H7 numbers on spinach (set A) treated with encapsulated eugenol micelles, free eugenol, empty micelles, chlorine, and water. Samples were treated via 2 min immersion and were stored at 5°C for up to 10 days**.** Lines depict means from duplicate triplicate identical replications (n = 6) while error bars depict standard deviation from the mean. Encapsulated eugenol, 1%SDS + 1% eugenol; free eugenol, 1%eugenol; empty micelles ,1%SDS; chlorine, 200 ppm chlorine. The limit of detection is 0.5 $\log_{10}$ CFU/cm^2.

levels of *S.* Saintpaul from day 3 to 10. From day 5 to 10, reduced populations of *S.* Saintpaul were observed with encapsulated eugenol, free eugenol, and chlorine ($p<0.05$) but not with water and empty micelles ($p\geq0.05$). Compared to the control, water treatment increased the population of *S.* Saintpaul to 4.7±0.3 $\log_{10}$ CFU/cm^2 on day 10. Compared to the level of *S.* on day 0, the levels of *S.* Saintpaul on the positive control decreased from day 5 to 10 of storage ($p<0.05$).

Figure 11-9b depicts populations of *E. coli* O157:H7 on spinach samples (set A) after treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water. The initial population of *E. coli* O157:H7 on the positive control on day 0 was 6.0±0.0 $\log_{10}$ CFU/cm^2. On day 0, all treatments excepting water reduced populations of *S.* Saintpaul to 2.2±1.0 to 5.0±0.3 $\log_{10}$ CFU/cm^2; free eugenol was the most effective among all treatments. From day 3 to 10, treatments resulted in *E. coli* O157:H7 populations ranging from 0.2±0.0 to 5.0±0.2 $\log_{10}$ CFU/cm^2. On day 3, populations of *E. coli* O157:H7 after all treatments were lower than the control. Encapsulated eugenol, free eugenol and chlorine were similarly effective ($p\geq0.05$) in reducing *E. coli* O157:H7 populations and were more efficient than empty micelles and water ($p<0.05$) on day 3. From day 3 to 10, encapsulated and free eugenol treatments produced undetectable levels of *E. coli* O157:H7 on spinach. From day 5 to 10, all treatments but water reduced populations of *E. coli* O157:H7 to lower levels than positive controls ($p<0.05$). The levels of *E. coli* O157:H7 on untreated spinach samples decreased to 4.6±0.7, 4.4±0.3 and 4.0±0.4 $\log_{10}$ CFU/cm^2 on day 5, 7 and 10 respectively.

Populations of *S.* Saintpaul and *E. coli* O157:H7 on spinach samples (set B) after

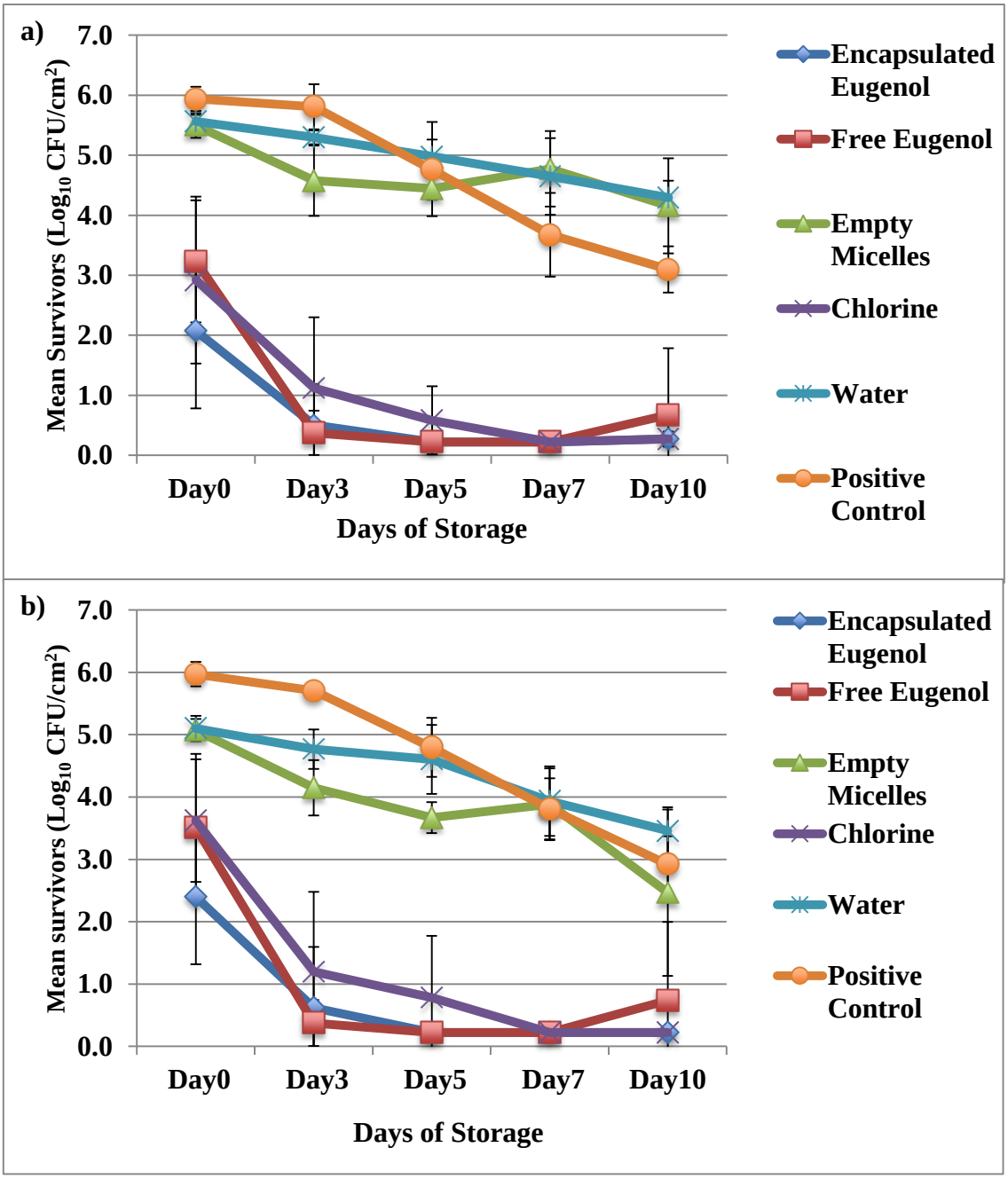

FIGURE 11-10. Survival of a) *Salmonella* Saintpaul and b) *Escherichia coli* O157:H7 numbers on spinach (set B) treated with encapsulated eugenol micelles, free eugenol, empty micelles, chlorine, and water. Samples were treated via 2 min immersion and were initially stored at 5°C. On day 5, samples were transitioned to 15°C. Lines depict means from duplicate triplicate identical replications (n = 6) while error bars depict standard deviation from the mean. Encapsulated eugenol, 1%SDS + 1% eugenol; free eugenol, 1% eugenol; empty micelles, 1% SDS; chlorine, 200 ppm chlorine. The limit of detection is 0.5 $\log_{10}$ CFU/cm^2.

treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water are shown in Figure 11-10a and 11-10b respectively. After treatments, set B of tomato samples was initially stored at 5°C and was transitioned to 15°C on day 5 of storage. The population of *S.* Saintpaul and *E. coli* O157:H7 on the positive controls were 5.9±0.2 and 6.0±0.2 $\log_{10}$ CFU/cm^2 respectively on day 0. Treatments with encapsulated eugenol, free eugenol, empty micelles, chlorine, and water on day 0 yielded *S.* Saintpaul and *E. coli* O157:H7 populations ranging from 2.1±1.3 to 5.6±0.1 $\log_{10}$ CFU/cm^2 and 2.4±1.1 to 5.1±0.2. $\log_{10}$ CFU/cm^2 respectively. Throughout 10 days of storage, encapsulated eugenol, free eugenol, and chlorine significantly reduced populations of *S.* Saintpaul and *E. coli* O157:H7 with undetectable levels observed after day 3 ($p<0.05$); the antimicrobial effects of encapsulated eugenol, free eugenol, and chlorine did not differ after day 3 ($p\geq0.05$). Water and empty micelles did not reduce levels of both pathogens after day 5 ($p\geq0.05$). On day 7 and 10, water and empty micelles treatments even yielded higher numbers of *S.* Saintpaul compared to controls ($p<0.05$). Compared to the initial levels of *S.* Saintpaul and *E. coli* O157:H7 on day 0, the levels of both pathogens significantly decreased after day 3 of storage ($p<0.05$).

Table 11-3 shows populations of *S.* Saintpaul and *E. coli* O157:H7 on set A and B of spinach samples. On day 7, excepting empty micelles, populations of both pathogens on set A did not differ from populations on set B samples ($p\geq0.05$). Compared to set A, treatment with empty micelles resulted in higher levels of both pathogens on set B samples on day 7 ($p<0.05$). On day 10, populations of both pathogens on set B of control samples were lower than populations on set A control samples ($p<0.05$). Higher

TABLE 11-3. Survival of *Salmonella* Saintpaul and *Escherichia coli* O157:H7 (log_{10} CFU/cm^2) on set A and B of spinach samples[a] after treatment with encapsulated eugenol, empty micelles, chlorine, and water.[b]

Treatment	Days of Storage	*Salmonella* Saintpaul		*Escherichia coli* O157:H7	
		Set A	Set B	Set A	Set B
Encapsulated Eugenol	0	2.8 ± 0.4^{n}	2.1 ± 1.3^{o}	3.1 ± 0.3^{lmn}	2.4 ± 1.1^{op}
Free Eugenol	0	1.8 ± 1.2^{op}	3.2 ± 1.0^{lmn}	2.2 ± 1.0^{op}	3.5 ± 0.9^{jklm}
Empty Micelles	0	5.4 ± 0.5^{abcd}	5.5 ± 0.2^{abc}	5.0 ± 0.2^{cde}	5.1 ± 0.2^{bcd}
Chlorine	0	2.0 ± 1.1^{o}	2.9 ± 1.4^{n}	2.7 ± 1.1^{no}	3.6 ± 1.0^{jkl}
Water	0	5.6 ± 0.3^{abc}	5.6 ± 0.1^{abc}	5.3 ± 0.5^{abc}	5.1 ± 0.2^{bcd}
Positive Control	0	6.0 ± 0.1^{a}	5.9 ± 0.2^{a}	6.0 ± 0.0^{a}	6.0 ± 0.2^{a}
Encapsulated Eugenol	3	0.2 ± 0.0^{r}	0.5 ± 0.7^{qr}	0.2 ± 0.0^{t}	0.6 ± 1.0^{st}
Free Eugenol	3	0.2 ± 0.0^{r}	0.4 ± 0.4^{r}	0.2 ± 0.0^{t}	0.4 ± 0.4^{t}
Empty Micelles	3	4.7 ± 0.5^{efgh}	4.6 ± 0.6^{fghi}	4.1 ± 0.3^{ghijk}	4.1 ± 0.4^{fghijk}
Chlorine	3	0.5 ± 0.7^{qr}	1.1 ± 1.2^{pq}	0.6 ± 0.9^{st}	1.2 ± 1.3^{qrs}
Water	3	5.2 ± 0.2^{bcdef}	5.3 ± 0.1^{abcde}	4.7 ± 0.3^{cdefg}	4.8 ± 0.1^{cdefg}
Positive Control	3	5.8 ± 0.2^{ab}	5.8 ± 0.4^{ab}	5.8 ± 0.2^{a}	5.7 ± 0.1^{ab}
Encapsulated Eugenol	5	0.2 ± 0.0^{r}	0.2 ± 0.0^{r}	0.2 ± 0.0^{t}	0.2 ± 0.0^{t}
Free Eugenol	5	0.2 ± 0.0^{r}	0.2 ± 0.0^{r}	0.2 ± 0.0^{t}	0.2 ± 0.0^{t}
Empty Micelles	5	4.4 ± 0.2^{ghij}	4.4 ± 0.5^{ghij}	3.8 ± 0.5^{ijkl}	3.7 ± 0.2^{ijkl}
Chlorine	5	1.5 ± 1.2^{op}	0.6 ± 0.6^{qr}	1.4 ± 1.1^{qr}	0.8 ± 1.0^{rst}
Water	5	4.8 ± 0.3^{defgh}	5.0 ± 0.6^{cdefg}	4.2 ± 0.3^{fghij}	4.6 ± 0.6^{defgh}
Positive Control	5	4.5 ± 0.8^{ghi}	4.8 ± 0.5^{defgh}	4.6 ± 0.7^{cdefgh}	4.8 ± 0.5^{cdef}
Encapsulated Eugenol	7	0.2 ± 0.0^{r}	0.2 ± 0.0^{r}	0.2 ± 0.0^{t}	0.2 ± 0.0^{t}
Free Eugenol	7	0.4 ± 0.3^{r}	0.2 ± 0.0^{r}	0.5 ± 0.6^{t}	0.2 ± 0.0^{t}
Empty Micelles	7	4.0 ± 0.4^{ijk}	4.8 ± 0.6^{defgh}	2.9 ± 0.4^{mno}	3.9 ± 0.6^{ijk}
Chlorine	7	0.8 ± 1.2^{qr}	0.2 ± 0.0^{r}	0.7 ± 1.1^{st}	0.2 ± 0.0^{t}
Water	7	4.8 ± 0.5^{defgh}	4.6 ± 0.6^{efgh}	4.1 ± 0.3^{ghijk}	3.9 ± 0.6^{ijk}
Positive Control	7	4.3 ± 0.2^{hijk}	3.7 ± 0.7^{klm}	4.4 ± 0.3^{efghi}	3.8 ± 0.5^{ijkl}
Encapsulated Eugenol	10	0.3 ± 0.1^{r}	0.3 ± 0.1^{r}	0.2 ± 0.0^{t}	0.2 ± 0.0^{t}
Free Eugenol	10	0.2 ± 0.0^{r}	0.7 ± 1.1^{qr}	0.2 ± 0.0^{t}	0.7 ± 1.3^{rst}
Empty Micelles	10	3.6 ± 0.4^{klm}	4.2 ± 0.8^{hijk}	1.7 ± 0.8^{pq}	2.5 ± 1.3^{no}
200 ppm Chlorine	10	0.4 ± 0.3^{r}	0.3 ± 0.1^{r}	0.4 ± 0.5^{t}	0.2 ± 0.0^{t}
Water	10	4.7 ± 0.3^{defgh}	4.3 ± 0.3^{hijk}	3.9 ± 0.3^{hijk}	3.5 ± 0.4^{klm}
Positive Control	10	3.8 ± 0.5^{jkl}	3.1 ± 0.4^{mn}	4.0 ± 0.4^{hijk}	2.9 ± 0.5^{mno}

[a]After treatments, set A of spinach samples was stored at 5°C for the entire 10-day storage period. Set B of spinach samples was initially stored at 5°C after treatments and were transitioned to 15°C on day 5 until day 10.

[b]Data represent means ± standard deviations from duplicate triplicate identical replications (n = 6). Across both sample sets, means within the same pathogen grouping not connected by same letter are significantly different ($p<0.05$). The limit of detection is 0.5 log_{10} CFU/cm^2.

population of *E. coli* O157:H7 on set B of spinach was observed with empty micelles treatment on day 10 of storage ($p<0.05$). Other treatments did not yield differences in population of *S.* Saintpaul and *E. coli* O157:H7 between set A and B on day 10 ($p\geq0.05$).

On day 0, initial populations of aerobic bacteria, Enterobacteriaceae, and yeasts and molds on untreated spinach sample (set A) were 5.1±0.6, 3.9±0.6, and 3.1±0.3 $\log_{10}$ CFU/cm^2 respectively (Figure 11-11a to c). Encapsulated eugenol and chlorine treatments reduced aerobic bacteria levels on day 0 to 3.2±0.7 and 3.0±2.0 $\log_{10}$ CFU/cm^2 respectively ($p<0.05$) while other treatments did not yield reduced populations of aerobic bacteria ($p\geq0.05$) (Figure 11-11a). From day 3 to 10, treatments with encapsulated eugenol, free eugenol, chlorine, empty micelles, and water resulted in populations of aerobic bacteria ranging from 2.6±0.7 to 5.1±1.1 $\log_{10}$ CFU/cm^2. On day 3 and 5, encapsulated eugenol, free eugenol, and chlorine significantly reduced population of aerobic bacteria on spinach ($p<0.05$) and their antimicrobial effects did not differ from each other ($p\geq0.05$). On day 7, reduction of aerobic bacteria levels was observed with encapsulated eugenol and free eugenol ($p<0.05$) but not with other treatments. All treatments significantly failed to reduce populations of aerobic bacteria on spinach on day 10 ($p\geq0.05$). On day 7 and 10, treatment with empty micelles increased populations of aerobic bacteria on spinach samples compared to the control levels ($p<0.05$). The levels of aerobic bacteria on the controls remained unchanged during 10-day storage period. For Enterobacteriaceae, on day 0, encapsulated eugenol, chlorine, and free eugenol significantly decreased the populations to the range of 1.9±1.0 to 2.3±1.1 $\log_{10}$ CFU/cm^2 ($p<0.05$) and their antimicrobial effects were not different

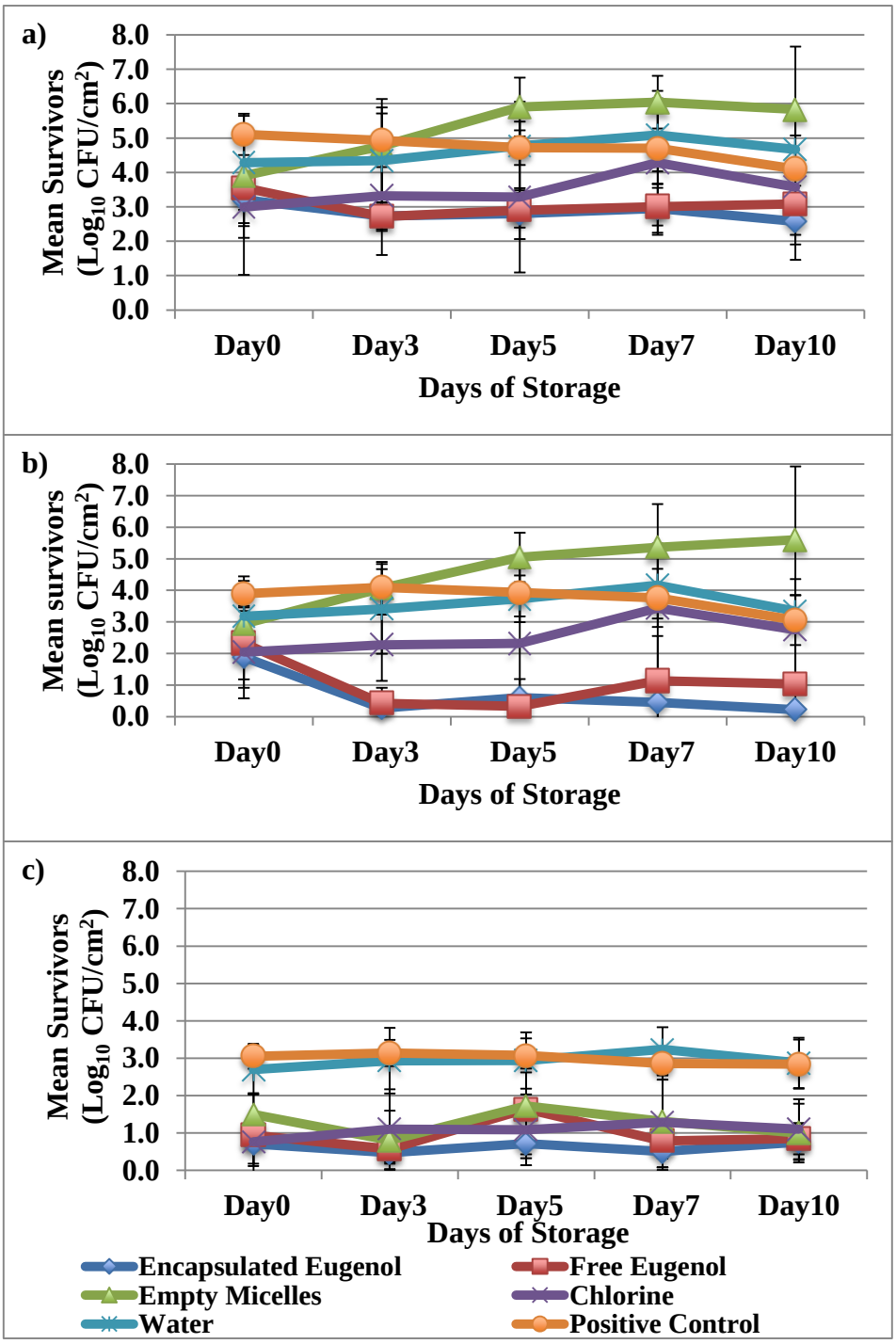

FIGURE 11-11. Survival of a) aerobic bacteria, b) Enterobacteriaceae, and c) yeasts and molds on spinach (set A) treated with encapsulated eugenol micelles, free eugenol, empty micelles, chlorine, and water. Samples were treated via 2 min immersion and were stored at 5°C for up to 10 days. Lines depict means from duplicate triplicate identical replications (n = 6) while error bars depict standard deviation from the mean. The limit of detection is 0.5 $\log_{10}$ CFU/cm^2.

from each other ($p \geq 0.05$) (Figure 11-11b). From day 3 to 10, populations of Enterobacteriaceae on spinach samples after treatments ranged from 0.2±0.0 to 5.6±2.3 $\log_{10}$ CFU/cm^2. Decreased levels of Enterobacteriaceae were observed with encapsulated eugenol, free eugenol, and chlorine ($p<0.05$) but not with water and empty micelles ($p \geq 0.05$) on day 3 to 5. After day 5 of storage, decreased levels of Enterobacteriaceae were observed with encapsulated eugenol and free eugenol treatments ($p<0.05$) but not with chlorine, water and empty micelles treatments ($p \geq 0.05$). On day 7 and 10, treatment with empty micelles resulted in higher numbers of Enterobacteriaceae compared to the controls. Numbers of Enterobacteriaceae on untreated samples did not differ throughout the entire storage period ($p \geq 0.05$). From day 0 to 10, populations of yeasts and molds after all treatments ranged from 0.5±0.3 to 3.2±0.6 $\log_{10}$ CFU/cm^2 (Figure 11-11c). Throughout 10 days of storage, all treatments except water significantly decreased populations of yeasts and molds on spinach samples ($p<0.05$). On day 0, 3, 7 and 10, the antifungal effects of encapsulated eugenol, free eugenol, empty micelles, and chlorine did not differ ($p \geq 0.05$). On day 5, antifungal effects of encapsulated eugenol, chlorine and free eugenol did not differ ($p \geq 0.05$) but encapsulated eugenol was more effective than empty micelles ($p<0.05$). The levels of yeasts and molds on untreated spinach samples did not differ throughout 10 days of storage ($p \geq 0.05$).

Figure 11-12 depicts populations of aerobic bacteria, Enterobacteriaceae, and yeasts and molds on spinach samples (set B). Initial populations of aerobic bacteria, Enterobacteriaceae, and yeasts and molds on day 0 were 5.0±0.7, 3.8±1.1, and 2.9±0.5 $\log_{10}$ CFU/cm^2 respectively. From day 0 to 10, populations of aerobic bacteria after all

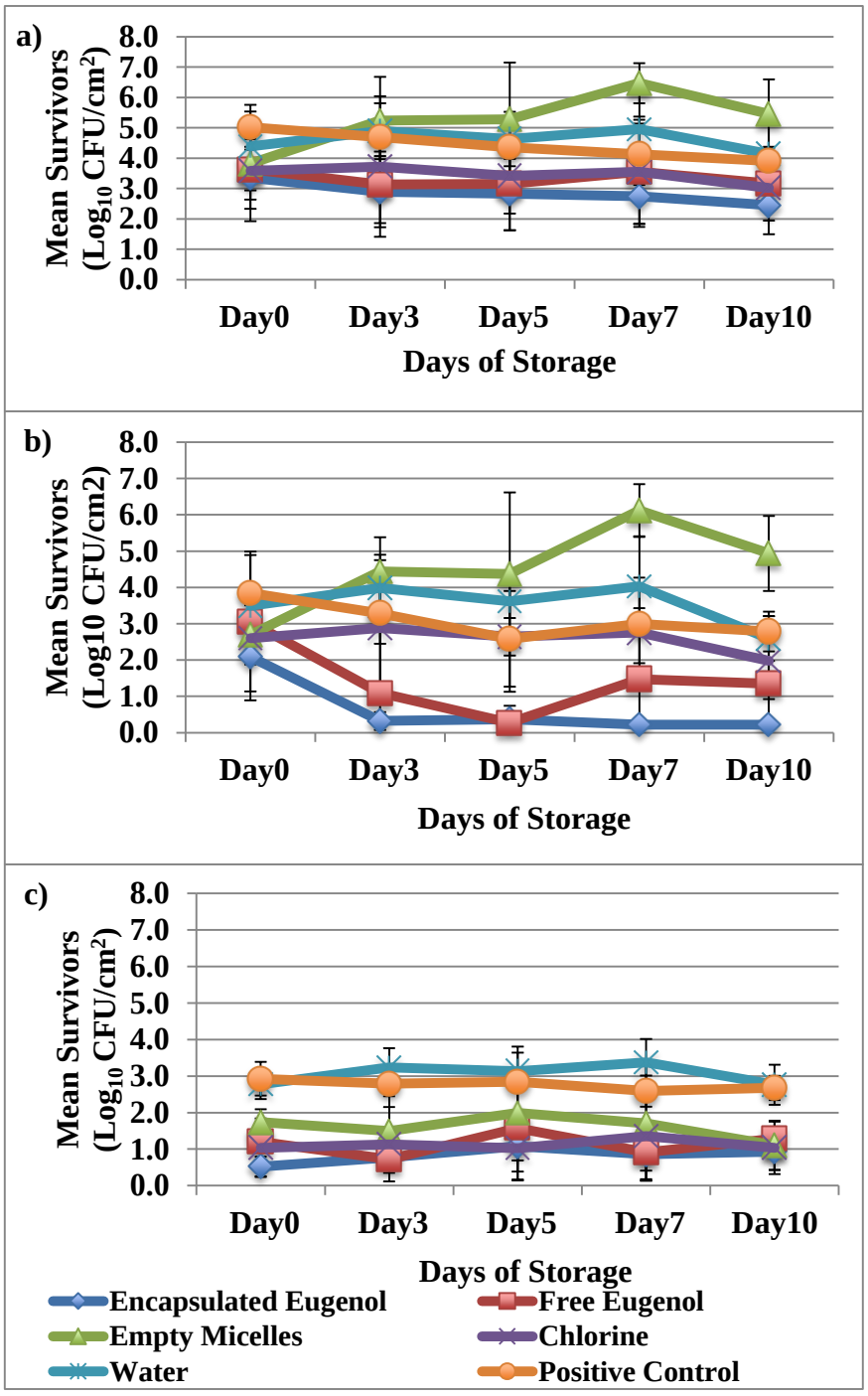

FIGURE 11-12. Survival of a) aerobic bacteria, b) Enterobacteriaceae, and c) yeasts and molds on spinach (set B) treated with encapsulated eugenol micelles, free eugenol, empty micelles, chlorine, and water. Samples were treated via 2 min immersion and were initially stored at 5°C and transitioned to 15°C on day 5. Lines depict means from duplicate triplicate identical replications (n = 6) while error bars depict standard deviation from the mean. The limit of detection is 0.5 $\log_{10}$ CFU/cm^2.

treatments ranged from 2.5±1.0 to 6.5±0.7 $\log_{10}$ CFU/cm^2. On day 0, encapsulated eugenol, free eugenol, and chlorine decreased populations of aerobic bacteria ($p<0.05$) and their effects did not differ from each other (Figure 11-12a). On day 3, only encapsulated eugenol reduced populations of aerobic bacteria ($p<0.05$). Reduction of aerobic bacteria levels on day 5 was observed with encapsulated eugenol and free eugenol treatments ($p<0.05$). On day 7 of storage, all treatments except encapsulated eugenol failed to decrease levels of aerobic bacteria on spinach samples ($p\geq0.05$). On day 10, only encapsulated eugenol significantly lowered the numbers of aerobic bacteria to 2.5±1.0 $\log_{10}$ CFU/cm^2. After day 5 of storage, empty micelles significantly increased populations of aerobic bacteria compared to controls ($p<0.05$). Levels of aerobic bacteria on positive controls did not significantly change over 10 days of storage. From day 0 to 10 of storage, populations of Enterobacteriaceae on spinach after all treatments ranged from 0.2±0.0 to 6.1±0.7 $\log_{10}$ CFU/cm^2 (Figure 11-12b). On day 0, reduction of Enterobacteriaceae was only observed with encapsulated eugenol. From day 3 to 10 of storage, treatments with free and encapsulated eugenol significantly reduced levels of Enterobacteriaceae ($p<0.05$) while other treatments did not inactivate Enterobacteriaceae ($p\geq0.05$). Empty micelles significantly increased populations of Enterobacteriaceae after day 3 of storage ($p<0.05$). Levels of Enterobacteriaceae on untreated samples remained unchanged over 10 days of storage ($p\geq0.05$). For yeasts and molds, after treatments, populations ranged from 0.5±0.4 to 3.2±0.6 $\log_{10}$ CFU/cm^2 (Figure 11-12c) On day 0, 3, and 10, reduction of Enterobacteriaceae was observed with all treatments except for water. Encapsulated eugenol, free eugenol, and chlorine were similarly effective in

reducing Enterobacteriaceae populations on day 5 and 7 ($p \geq 0.05$) while empty micelles and water did not show inhibitory effects ($p \geq 0.05$). The levels of yeasts and molds on the positive controls did not change over 10 days of storage ($p \geq 0.05$).

Table 11-4 represents populations of aerobic bacteria, Enterobacteriaceae, and yeasts and molds on spinach samples (set A and B). On day 7 and 10, populations of each type of native microbiota on treated and untreated samples did not differ between two sample sets ($p \geq 0.05$). Overall, for pathogen reduction on spinach surfaces, the trend of antimicrobial effect from greatest to least was encapsulated eugenol = free eugenol = chlorine > empty micelles ≥ water. For aerobic bacteria and Enterobacteriaceae on spinach samples, treatments followed the trend from greatest to least antibacterial effect of encapsulated eugenol = free eugenol ≥ chlorine > water > SDS. The antifungal effect of treatments on spinach surfaces followed the trend of encapsulated eugenol = free eugenol = chlorine = empty micelles > water. Encapsulated eugenol, free eugenol, and chlorine exerted efficient residual effects in reducing pathogen populations to below or just over detectable levels after day 0 of storage. The rough surface of spinach *(262)* as well as cracks, pockets, crevices and native openings (e.g., stomata) may favor microbial attachment and provide protection to microorganisms from antimicrobial intervention *(245, 263)*. On leaf surfaces, there is a boundary layer, which is a thin layer of air influenced by the leaf surface *(153)*. The layer can vary from less than 1 mm to 10 mm thick and can influence the temperature, moisture, and speed of air movement *(153)*. When spinach samples were treated with encapsulated and free eugenol, the

TABLE 11-4. Survival of aerobic bacteria, Enterobacteriaceae, and yeasts and molds (log_{10} CFU/cm^2) on set A and B of spinach samples[a] after treatment with encapsulated eugenol, empty micelles, chlorine, and water.[b]

Treatment	Days of Storage	Aerobic Bacteria		Enterobacteriaceae		Yeasts and Molds	
		Set A	Set B	Set A	Set B	Set A	Set B
Encapsulated Eugenol	0	3.2 ± 0.7^{nopqrst}	3.4 ± 1.0^{klmnopqrst}	1.9 ± 1.0^{qrstu}	2.1 ± 1.2^{nopqrst}	0.7 ± 0.5^{hij}	0.5 ± 0.3^{ij}
Free Eugenol	0	3.6 ± 1.5^{ijklmnopqrst}	$3.6\pm1.0^{ghijklmnopqrst}$	2.3 ± 1.1^{lmnopqrst}	3.0 ± 0.9^{ghijklmnopq}	0.9 ± 1.1^{defghij}	1.2 ± 0.3^{cdefghij}
Empty Micelles	0	$3.9\pm1.5^{efghijklmnopqrs}$	$3.8\pm0.9^{fghijklmnopqrst}$	2.9 ± 0.9^{hijklmnopq}	2.7 ± 0.8^{jklmnopqr}	1.5 ± 0.5^{cdefgh}	1.7 ± 0.4^{cd}
Chlorine	0	3.0 ± 2.0^{nopqrst}	$3.6\pm1.7^{ghijklmnopqrst}$	2.0 ± 1.5^{opqrst}	2.6 ± 1.5^{klmnopqrs}	0.8 ± 0.6^{ghij}	1.0 ± 0.8^{defghij}
Water	0	$4.3\pm1.4^{defghijklmnop}$	4.4 ± 1.1^{defghijklmn}	3.2 ± 1.1^{fghijklmnopq}	3.5 ± 1.4^{fghijklm}	2.7 ± 0.7^{ab}	2.8 ± 0.4^{ab}
Positive Control	0	5.1 ± 0.6^{abcdef}	5.0 ± 0.7^{bcdefg}	3.9 ± 0.6^{defghijk}	3.8 ± 1.1^{defghijk}	3.1 ± 0.3^{a}	2.9 ± 0.5^{a}
Encapsulated Eugenol	3	2.7 ± 0.4^{qrst}	2.9 ± 1.2^{pqrst}	0.3 ± 0.1^{v}	0.3 ± 0.2^{v}	0.5 ± 0.3^{j}	0.8 ± 0.2^{ghij}
Free Eugenol	3	2.7 ± 0.4^{rst}	3.1 ± 1.3^{nopqrst}	0.4 ± 0.5	1.1 ± 2.1^{tuv}	0.6 ± 0.4^{ij}	0.7 ± 0.4^{ghij}
Empty Micelles	3	4.8 ± 1.4^{defghij}	5.2 ± 1.4^{abcde}	4.1 ± 0.8^{cdefghi}	4.4 ± 0.9^{bcdef}	0.8 ± 0.8^{fghij}	1.5 ± 1.1^{cdefgh}
Chlorine	3	3.3 ± 1.7^{lmnopqrst}	$3.7\pm2.3^{fghijklmnopqrst}$	2.3 ± 1.1^{mnopqrst}	2.9 ± 1.9^{hijklmnopq}	1.1 ± 1.1^{defghij}	1.1 ± 1.0^{cdefghij}
Water	3	4.3 ± 1.6^{defghijklmno}	4.9 ± 0.9^{bcdefghi}	3.4 ± 1.4^{fghijklmn}	4.0 ± 0.9^{defghij}	2.9 ± 0.9^{a}	3.2 ± 0.5^{a}

TABLE 11-4. Continued.

Treatment	Days of Storage	Aerobic Bacteria		Enterobacteriaceae		Yeasts and Molds	
		Set A	Set B	Set A	Set B	Set A	Set B
Positive Control	3	4.9±0.8^{bcdefghi}	4.7±0.5^{bcdefghijkl}	4.1±0.6^{cdefghi}	3.3±0.8^{fghijklmnop}	3.1±0.4^{a}	2.8±0.3^{ab}
Encapsulated Eugenol	5	2.8±0.7^{qrst}	2.8±1.2 ^{qrst}	0.6±0.6^{uv}	0.4±0.4^{v}	0.7±0.4^{ghij}	1.1±0.4^{defghij}
Free Eugenol	5	2.9±0.5^{pqrst}	3.2±1.0^{nopqrst}	0.3±0.2^{v}	0.3±0.1^{v}	1.6±1.2^{cdefg}	1.6±1.2^{cdefg}
Empty Micelles	5	5.9±0.9^{abc}	5.3±1.9^{abcde}	5.0±0.8^{abcd}	4.4±2.2^{bcdef}	1.7±1.0^{cd}	2.0±1.8^{bc}
Chlorine	5	3.3±2.2^{mnopqrst}	3.4±1.8^{jklmnopqrst}	2.3±1.8^{lmnopqrst}	2.6±1.5^{klmnopqrs}	1.1±0.9^{defghij}	1.0±0.9^{defghij}
Water	5	4.8±1.3^{bcdefghij}	4.6±0.9^{cdefghijklm}	3.7±0.7^{defghijk}	3.6±0.5^{efghijkl}	2.9±0.8^{a}	3.1±0.5^{a}
Positive Control	5	4.7±0.5^{bcdefghijk}	4.4±0.4^{defghijklmn}	3.9±0.8^{defghijk}	2.6±1.3^{klmnopqrs}	3.1±0.5^{a}	2.8±0.2^{a}
Encapsulated Eugenol	7	3.0±0.7^{opqrst}	2.7±0.9^{qrst}	0.5±0.6^{v}	0.2±0.0^{v}	0.5±0.4^{j}	0.9±0.7^{efghij}
Free Eugenol	7	3.0±0.5^{nopqrst}	3.6±1.8^{ijklmnopqrst}	1.1±1.4^{tuv}	1.5±2.0^{rstuv}	0.8±0.5^{ghij}	0.9±0.5^{defghij}
Empty Micelles	7	6.0±0.8^{ab}	6.5±0.7^{a}	5.4±1.4^{abc}	6.1±0.7^{a}	1.3±1.2^{cdefghij}	1.7±1.6^{cde}
Chlorine	7	4.3±2.1^{defghijklmnop}	3.6±1.7^{ijklmnopqrst}	3.4±2.0^{fghijklm}	2.8±1.5^{ijklmnopqr}	1.3±1.3^{cdefghij}	1.4±1.2^{cdefghi}
Water	7	5.1±1.1^{abcdef}	5.0±1.4^{bcdefgh}	4.2±1.1^{cdefgh}	4.0±1.4^{cdefghi}	3.2±0.6^{a}	3.4±0.6^{a}

TABLE 11-4. Continued.

Treatment	Days of Storage	Aerobic Bacteria		Enterobacteriaceae		Yeasts and Molds	
		Set A	Set B	Set A	Set B	Set A	Set B
Positive Control	7	4.7±1.0$^{bcde\ fghijklm}$	4.1±1.0$^{defg\ hijklmnopq}$	3.8±0.9$^{defg\ hijk}$	3.0±1.1$^{hijkl\ mnopq}$	2.9±0.4^{a}	2.6±0.4^{ab}
Encapsulated Eugenol	10	2.6±0.7st	2.5±1.0^{t}	0.2±0.0^{v}	0.2±0.0^{v}	0.8±0.5^{ghij}	0.9±0.5$^{defg\ hij}$
Free Eugenol	10	3.1±0.9$^{nopq\ rst}$	3.2±0.9$^{nopq\ rst}$	1.0±1.8^{tuv}	1.3±1.9^{stuv}	0.8±0.4$^{efghi\ j}$	1.3±0.1$^{cdef\ ghij}$
Empty Micelles	10	5.8±1.8^{abc}	5.5±1.1^{abcd}	5.6±2.3^{ab}	4.9±1.0$^{abcd\ e}$	1.0±0.8$^{defg\ hij}$	1.1±0.7$^{defg\ hij}$
Chlorine	10	3.6±2.1$^{hijkl\ mnopqrst}$	3.0±1.1$^{nopq\ rst}$	2.8±1.6$^{ijklm\ nopqr}$	2.0±1.1^{pqrst}	1.1±0.8$^{defg\ hij}$	1.0±0.7$^{defg\ hij}$
Water	10	4.7±1.1$^{bcde\ fghijklm}$	4.1±1.0$^{defg\ hijklmnopq}$	3.3±0.5$^{fghij\ klmno}$	2.6±0.6$^{klmn\ opqrs}$	2.9±0.7^{a}	2.8±0.6^{ab}
Positive Control	10	4.1±1.0$^{defg\ hijklmnopqr}$	3.9±0.5$^{efghi\ jklmnopqrs}$	3.1±0.8$^{ghijk\ lmnopq}$	2.8±0.5$^{ijklm\ nopqr}$	2.8±0.7^{a}	2.7±0.3^{ab}

[a]After treatments, set A of spinach samples was stored at 5°C for the entire 10-day storage period. Set B of spinach samples was initially stored at 5°C after treatments and were transitioned to 15°C on day 5 until day 10.
[b]Data represent means ± standard deviations from duplicate triplicate identical replications (n = 6). Across both sample sets, means within the same pathogen grouping not connected by same letter are significantly different ($p<0.05$). The limit of detection is 0.5 $\log_{10}$ CFU/cm^2.

antimicrobials may have been trapped in a boundary layer and crevices. During storage, eugenol may have vaporized and exerted residual effect in inactivating microorganisms. The surface of spinach is covered with cuticle, a continuous extracellular membrane polymerized lipids with associated waxes *(126)*. The hydrophobic nature of the waxy cuticle may have prevented chlorine from reducing microorganisms on spinach surfaces. Thus, in this study, chlorine inactivated Enterobacteriaceae less effectively than encapsulated and free eugenol. Effects of chlorine on microbial inactivation in leafy greens have been reported. Zhang and Farber *(264)* reported the maximum $\log_{10}$ reduction of *L. monocytogenes* at 4 and 22°C to be 1.3 log and 1.7 $\log_{10}$ CFU/g for lettuce and 0.9 and 1.2 $\log_{10}$ CFU/g for cabbage respectively. Chlorine tested in combination with individual surfactants (Orenco Peel 40 and Tergitol) did not yield more reduction of *L. monocytogenes (264)*. Erkman *(82)* reported that 10 ppm of HOCL (pH 7.0) applied via immersion with agitation for 5 min reduced *E. coli* on lettuce, parsley and pepper by 1.23, 1.61, and 2.64 $\log_{10}$ CFU/ml respectively. Commonly used concentrations (50-200 ppm) with 1 to 2 min contact time normally produced a maximum 1 to 2 $\log_{10}$ reduction of many commodities *(90)*. In produce packing lines, accumulation of organic matter (e.g. field soil, debris, fruit, leaves) in dump tank or flume water as well as alkaline pH of wash water can decrease effectiveness of chlorine *(33, 59)*. In this study, empty micelles (1% SDS) failed to reduce pathogen levels at 15°C and even increased levels of aerobic bacteria and Enterobacteriaceae after day 5 of storage at both 5°C and 15°C. SDS is a derivative of lauric acid and is a mixture of sodium alkyl sulfates consisting of a 12-carbon tail attached to a sulfate head group,

rendering it amphiphilic *(139, 213)*. Enterobacteriaceae and pseudomonads are predominant on surfaces of leafy greens *(153)*; thus, increased populations of aerobic bacteria and Enterobacteriaceae on spinach surfaces could have been due to the ability of these bacteria to metabolize or tolerate SDS *(103, 147, 226)*. Kramer et al. *(147)* reported that 200 strains of independent isolates of Enterobacteriaceae (e.g. *E. coli*, *Shigella flexneri*, *Shigella sonnei*, *Salmonella* Arizonae, *Klebsiella pneumoniae* etc.) were highly resistant to SDS and were able to grow in the presence of ≥5% SDS. Thomas and white *(226)* proposed the complete metabolic pathway for degradation of SDS by *Pseudomonas* sp. C12B. Initially the sulfate group of SDS was cleaved by an alkyl sulfatase to produce 1-dodecanol which was then converted into dodecanal by an alcohol dehydrogenase enzyme. Dodecanal was further converted into dodecanoic acid by an aldehyde dehydrogenase. From this point, the pathway diverged into 1) beta oxidation which resulted in CO_2 liberation or 2) a process of elongation and saturation that produced C14, C16, and C18 saturated and unsaturated fatty acids which could be incorporated into membrane lipids of the microorganism *(226)*. In the current study. EOC did not show different antimicrobial effect at 5 and 15°C in both tomato and spinach. Mixed results of temperature on antimicrobial effect of EOCs were reported in previous studies. Nunez *(174)* reported that clove essential oil inactivated *E. coli*, *Staphylococcus aureus* and *P. aeruginosa* more effectively at 37°C than at 21°C. Increased activity of clove and cinnamon essential oils against *L. monocytogenes* in, pasteurized milk incubated at 7 versus 35°C was reported by Cava et al. *(43)*. In

response to low temperature, the degree of unsaturation of membrane lipid increased to maintain fluidity. With increased fluidity, EOCs may dissolve in the lipid bilayer of microorganisms more easily at low temperature versus higher temperature *(43)*. Overall, from the current study, encapsulated and unencapsulated EOC showed efficient antimicrobial effect and may be used as an alternative to conventional methods for pathogen decontamination in fresh produce commodities as well as increase produce shelf life.

11.2.4 Particle Sizes

The z-average diameter (the mean hydrodynamic diameter) and the polydispersity index (an estimate of the width of the size distribution) of encapsulated eugenol (1.0% SDS + 1.0% eugenol), free eugenol (1.0% eugenol), and empty micelles (1% SDS) are shown in Table 11-5. The particle size of encapsulated eugenol, free eugenol, and empty micelles were 534.9±221.7, 2231.0±380.7, 368.5±160.4 nm respectively. The particle size of free eugenol was greater than the sizes of encapsulated and free eugenol ($p<0.05$) while the sizes of encapsulated eugenol and free eugenol did not significantly differ ($p\geq0.05$). Gaysinsky et al. *(109)* studied the particle sizes of differing concentrations of carvacrol and eugenol encapsulated in 5% Surfynol® 485W micelles. The sizes of micelles increased as a function of concentration of EOCs *(109)*. Uluata *et al. (237)* reported particle sizes of 10% octadecane oil-in-water emulsions stabilized by 1% SDS after homogenization at 20 psi for 1 and 5 passes to be 230 and 102 nm respectively *(237)*.

11.2.5 ζ-potential

ζ-potential of encapsulated eugenol (1.0% SDS + 1.0% eugenol), free eugenol (1.0% eugenol), and empty micelles (1% SDS) are shown in Table 11-6. ζ-potential of encapsulated eugenol, free eugenol, and empty micelles were -65.8±9.5, -65.2±1.5, and -50.1±12.1 mv respectively. ζ-potential is the electrical potential at the shear plane, which is defined as the distance away from the droplet surface below which the counter-ions remain strongly attached to the droplet when it moves along the electric field *(162)*. Generally, particles with zeta potential more negative than -30 mV or more positive than +30 mV are considered stable *(191)*. In this study, ζ-potential of encapsulated eugenol, free eugneol, and empty micelles of were more negative than -30 mV and thus are considered stable. Normally, droplets stabilized by anionic surfactants have a negative charge while those stabilized by non-ionic and anionic surfactants tend to have only a small droplet charge and a positive charge respectively *(162)*.

TABLE 11-5. Means of z-average diameter (nm) and polydispersity index of encapsulated eugenol, free eugenol, and empty micelles.

Particle Sample	Z-Average	Polydispersity Index
Encapsulated Eugenol	534.9±221.7[b]	0.8±0.2
Free Eugenol	2231.0±380.7[a]	0.3±0.1
Empty Micelles	368.5±160.4[b]	0.8±0.1

Values represent means ± standard deviations from triplicate identical replications (n = 3). Means not connected by same letter are significantly different ($p<0.05$). Encapsulated eugenol, 1%SDS + 1% eugenol; free eugenol, 1% eugenol; empty micelles, 1% SDS .

TABLE 11-6. Means ζ-potential (mV) of encapsulated eugenol, free eugenol, and empty micelles.

Particle Sample	ζ-potential
Encapsulated Eugenol	-65.8±9.5[b]
Free Eugenol	-65.2±1.5[b]
Empty Micelles	-50.1±12.1[a]

Values represent means ± standard deviations from triplicate identical replications (n = 3). Means not connected by same letter are significantly different ($p<0.05$). Encapsulated eugenol, 1%SDS + 1% eugenol; free eugenol, 1% eugenol; empty micelles, 1% SDS.

CHAPTER XII

SUMMARY AND CONCLUSIONS

Quantification of native microbiota on leafy greens (lettuce, spinach, and parseley) from two farms in South Texas was conducted in spring harvest season while quantification of native microbiota on jalapeno pepper, tomato, and cantaloupe was conducted in spring and fall harvest seasons. For lettuce, populations of aerobic bacteria, total pseudomonads from PF agar, fluorescein-producing pseudomonads, total pseudomonads from PP agar, pyocyanin-producing pseudomonads, yeasts and molds, enterococci, total coliforms, *E. coli*, homofermentative LAB, and heterofermentative LAB from 2 farms ranged from 0.7±0.0 to 6.2±0.1 $\log_{10}$ CFU/g. For spinach and parsley, the populations of native microbiota from two farms ranged from 0.7±0.0 to 7.2±0.3 $\log_{10}$ CFU/g and 0.7±0.0 to 5.0±0.7 $\log_{10}$ CFU/g respectively. Overall, higher counts of certain microbial groupings were observed with leafy green samples collected at higher ambient temperature due to their mesophilic nature. Native microbiota on surfaces of jalapeno pepper, tomato, and cantaloupe obtained from spring and fall harvest seasons ranged from 0.2±0.0 to 3.9±0.7 $\log_{10}$ CFU/cm^2, 0.2±0.0 to 3.8±0.9 $\log_{10}$ CFU/cm^2, and 1.1±1.3 to 6.0±0.8 $\log_{10}$ CFU/cm^2 respectively. For stem scars, populations of native microbiota on tomato and cantaloupe obtained from two harvest seasons were in the ranged of 0.9±0.0 to 5.4±0.4 $\log_{10}$ CFU/cm^2 and 1.5±1.9 to 7.0±0.7 $\log_{10}$ CFU/cm^2 respectively. In tomatoes, higher counts of certain microbial groupings were observed in spring harvest season that was warmer than fall season. However, the same trend was not observed with cantaloupe and pepper, suggesting that other factors

also affect numbers of native microbiota. Overall, stem scars of tomato and cantaloupe bore greater counts of native microbiota versus skins/rinds. Porous nature as well as roughness of stem scars may favor attachment and also provide protection for microorganisms.

Dye internalization with aid of temperature and pressure differential through tomato stem scar of intact and nonintact tomatoes was studied as a representative of microbial internalization in tomato. Results showed that dye penetrations in intact and non-intact tomatoes were 1.71±1.36 cm and 0.10±0.06 cm respectively. This suggests that cutting tomato may have exposed the internal part of the fruit to the dye/environment resulting in reduced pressure differential between the fruit and a dye solution and thus prevent dye from infiltrating the fruit through stem scars efficiently. The study of microbial internalization without aid of temperature and pressure differential showed that internalization of *E. coli* K12 occurred through stem scar; however, *E. coli* K12 was unable to travel to cells deep within the stem probably due to hydrophobic nature of stem scars. SEM observation revealed vascular bundle structures with approximately 100 μm length and 15 μm diameter that are larger than bacterial sizes and thus could allow bacteria to infiltrate plant stem scars and move along vascular bundles.

The study of MAC of EOCs in surfactant micelles showed that the MACs of EOCs in micelles increased with increasing surfactant concentrations. Amongst micelles tested, carvacrol in Tween 20 micelles showed the lowest MAC and the highest MICs against *S.* Saintpaul and *E. coli* O157:H7. Conversely, micelles prepared from SDS and

CG20 containing eugenol and carvacrol were observed to produce the lowest MICs against pathogens. Carvacrol and eugenol encapsulated in SDS and CG20 micelles were most effective for pathogen inhibition *in vitro*, likely due to the combined antimicrobial activities of both EOC and surfactant, as well as increased loading of the EOC into micelles.

The antimicrobial effect of encapsulated eugenol, free eugenol, empty micelles, chlorine, and water was studied in tomato and spinach samples. Samples were treated with antimicrobials and were stored for 0, 3, 5, 7 and 10 days. Initially, all samples were stored at 5°C and one set of samples was shifted to 15°C on day 5. Overall, for pathogen inactivation on tomatoes, all treatments followed the trend from greatest to least antimicrobial effect of encapsulated eugenol = free eugenol = chlorine ≥ empty micelles > water. After day 0 storage, all treatments except water showed efficient residual effects in reducing pathogen populations to below or just over detectable levels of at 5°C for the entire storage period. Nevertheless, empty micelles was less effective in reducing pathogens on tomatoes at 15°C. For native microbiota, in general, all treatments excepting water were similarly effective in reducing aerobic bacteria and Enterobacteriaceae populations during storage at 5°C. At 15°C, chlorine was less effective than encapsulated eugenol, free eugenol, and empty micelles. For antifungal effect on tomatoes, treatments followed the trend of encapsulated eugenol > empty micelles ≥ free eugenol > chlorine = water. For pathogen reduction on spinach surfaces, overall, the trend of antimicrobial effect from greatest to least was encapsulated eugenol = free eugenol = chlorine > empty micelles ≥ water. For aerobic bacteria and

Enterobacteriaceae on spinach samples, treatments followed the trend from greatest to least antibacterial effect of encapsulated eugenol = free eugneol ≥ chlorine > water > SDS. The antifungal effect of treatments on spinach surfaces followed the trend of encapsulated eugenol = free eugenol = chlorine = empty micelles > water. Efficient residual effects in reducing pathogen populations on spinach after day 0 was observed with encapsulated eugenol, free eugenol, and chlorine. The study suggests that plant-derived antimicrobial EOCs loaded into food-grade surfactant micelles, as an alternative to conventional intervention methods, can be useful for the decontamination of fresh produce surfaces from cross-contaminating bacterial pathogens as well as extending fresh produce shelf life.

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